L8	L7 and (tumor$ or tumour$ or cancer$)	69	L8
L7	(cd59)same (transfect$ or transform$) same (cell$ or vector$)	88	L7
L6	L5 same (tumor$ or cancer$ or tumour$)	24	L6
L5	(cd59)same (cd28 or ctla$ or b7$ or cd80 or cd86)	87	L5
L4	L3 same (transfect$ or transform$)	33	L4
L3	(cd59)same (tumor $ or tumour$ or cancer$)	71	L3
L2	L1 and cd59	2	L2
L1	boussiotis.in.	23	L1

END OF SEARCH HISTORY

Gambel, Phillip

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---------- cd59 ----------

9/7/4 (Item 4 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0012614997 BIOSIS NO.: 200000333310
CD59 expressed on a tumor cell surface modulates decay-accelerating factor expression and enhances tumor growth in a rat model of human neuroblastoma
AUTHOR: **Chen Shaohua; Caragine Theresa; Cheung Nai-Kong V; Tomlinson Stephen (Reprint)**
AUTHOR ADDRESS: Department of Microbiology and Immunology, Medical University of South Carolina, BSB 201, 173 Ashley Avenue, Charleston, SC, 29425, USA**USA
JOURNAL: **Cancer Research 60 (11): p3013-3018 June 1, 2000 2000**
MEDIUM: print
ISSN: 0008-5472
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: It has been hypothesized that complement inhibitors expressed on the surface of tumor cells prevent effective immune-mediated clearance. Whereas there are in vitro data to support this hypothesis, the species-selective activity of complement inhibitors has been a hindrance to investigating the role of membrane-bound complement inhibitors in rodent models of human cancer. The CD59-positive LAN-1 human neuroblastoma cell line was significantly more sensitive to lysis by rat complement than by human complement, illustrating the species selectivity of endogenously expressed complement inhibitors. Transfection of LAN-1 cells with rat CD59, an inhibitor of the terminal cytolytic membrane attack complex, effectively protected the cells from lysis by rat complement in vitro. When LAN-1 cells stably expressing rat CD59 were inoculated into immune-deficient rats, the onset of tumor growth and the rate of tumor growth were significantly enhanced compared with those of control-transfected LAN-1 cells. These data show directly that the expression of a complement inhibitor on a tumor cell promotes tumor growth. Flow cytometric analysis revealed that the endogenous expression of decay-accelerating factor (DAF), an inhibitor of complement activation, was up-regulated on the surface of cells after in vivo growth. Of further interest, higher levels

of DAF were present on CD59-transfected cells than on control-transfected cells derived from tumors. Increased DAF expression correlated with decreased complement deposition on the tumor cell surface. These results show that expression of complement inhibitors on a tumor cell has functional consequences with regard to complement deposition in vivo and indicate that CD59 can indirectly effect complement activation and C3 deposition in vivo via a link between CD59 and DAF expression.

---------- cd59 ----------

9/7/5 (Item 5 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0011828122 BIOSIS NO.: 199900087782
Protection of human breast cancer cells from complement-mediated lysis by expression of heterologous CD59
AUTHOR: **Yu J; Caragine T; Chen S; Morgan B P; Frey A B; Tomlinson Stephen (Reprint)**
AUTHOR ADDRESS: New York Univ. Med. Cent., Dep. Pathol., MSB 127, 550 First Avenue, New York, NY 10016, USA**USA
JOURNAL: **Clinical and Experimental Immunology 115 (1): p13-18 Jan., 1999 1999**
MEDIUM: print
ISSN: 0009-9104
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: CD59, decay accelerating factor (DAF) and membrane cofactor protein (MCP) are widely expressed cell surface glycoproteins that protect host cells from the effects of homologous complement attack. Complement inhibitory activity of these proteins is species-selective. We show that the human breast cancer cell line MCF7 is relatively resistant to lysis by human complement, but is effectively lysed by rat or mouse complement. CD59, DAF and MCP were all shown to be expressed by MCF7. The species-selective nature of CD59 activity was used to demonstrate directly the effectiveness of CD59 at protecting cancer cells from complement-mediated lysis. cDNAs encoding rat and mouse CD59 were separately transfected into MCF7 cells, and cell populations expressing high levels of the rodent CD59 were isolated by cell sorting. Data show that rat and mouse CD59 were highly effective at protecting transfected MCF7 cells from lysis by rat and mouse complement, respectively. Data further reveal that rat CD59 is not effective against mouse complement, whereas mouse CD59 is effective against both mouse and rat complement. These studies establish a model system for relevant in vivo studies aimed at determining the effect of complement regulation on tumorigenesis, and show that for effective immunotherapy using complement-activating anti-tumour antibodies, the neutralization of CD59 and/or other complement inhibitory molecules will probably be required.

---------- cd59 ----------

9/7/7 (Item 2 from file: 73)
DIALOG(R)File 73:EMBASE

12209865 EMBASE No: 2003322989
Obstacles to cancer immunotherapy: Expression of membrane complement

regulatory proteins (mCRPs) in tumors
Fishelson Z.; Donin N.; Zell S.; Schultz S.; Kirschfink M.
Z. Fishelson, Dept. of Cell/Developmental Biology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978 Israel
AUTHOR EMAIL: lifish@post.tau.ac.il
Molecular Immunology (MOL. IMMUNOL.) (United Kingdom) 2003, 40/2 (109-123)
CODEN: IMCHA ISSN: 0161-5890
DOCUMENT TYPE: Journal ; Conference Paper
LANGUAGE: ENGLISH SUMMARY LANGUAGE: ENGLISH
NUMBER OF REFERENCES: 209

Monoclonal antibodies (mAbs) are being increasingly used in cancer therapy owing to their ability to recognize specifically cancer cells and to activate complement- and cell-mediated cytotoxicity and/or to induce growth arrest or apoptosis. The therapeutic potential of anticancer antibodies is significantly limited due to the ability of cancer cells to block killing by complement. Of the multiple resistance strategies exploited by cancer cells, the expression of membrane complement regulatory proteins (mCRPs), such as CD46 (membrane cofactor protein (MCP)), CD55 (decay-accelerating factor (DAF)), CD35 (complement receptor type-1 (CR1)) and CD59, has received most attention. CD46, CD55 and CD35 block the complement cascade at the C3 activation stage and CD59 prevents assembly of the membrane attack complex of complement (MAC). These proteins protect normal tissues from accidental injury by activated complement, but also confer resistance on cancer cells, thereby limiting the effect of complement-fixing monoclonal antibodies. Expression of mCRPs on malignant cells is highly variable, yet there is clear indication that certain tumors express higher mCRP levels than the normal tissue from which they have evolved. mCRP level of expression and cellular location may also vary during malignant transformation and between differentiated and undifferentiated tumors. Neutralizing anti-mCRP mAbs have been used in vitro to elucidate the significance of mCRP expression to the tumor complement resistance phenotype. In general, CD59 appears to be the most effective mCRP protecting tumor cells from complement-mediated lysis. Nevertheless, it acts additively, and in certain tumors even synergistically, with CD55 and CD46. It is envisaged that treatment of cancer patients with mCRP blocking antibodies targeted specifically to cancer cells in combination with anticancer complement-fixing antibodies will improve the therapeutic efficacy. (c) 2003 Elsevier Ltd. All rights reserved.

--------- protectin -------

? t s13/7/4-10

13/7/4 (Item 4 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0009863458 BIOSIS NO.: 199598331291
Expression of protectin (CD59) in human melanoma and its functional role in cell- and complement-mediated cytotoxicity
AUTHOR: **Brasoveanu Lorelei I; Altomonte Maresa; Gloghini Annunziata; Fonsatti Ester; Coral Sandra; Gasparollo Aldo; Montagner Roberto; Cattarossi Ilaria; Simonelli Cecilia; Cattelan Alessandro; Attadia Vincenza; Carbone Antonino; Maio Michele (Reprint)**
AUTHOR ADDRESS: Advanced Immunotherapy Unit, INRCCS-CRO, Via Pedemontana Occ.le 12, Aviano, Italy**Italy
JOURNAL: **International Journal of Cancer 61 (4): p548-556 1995 1995**
ISSN: 0020-7136
DOCUMENT TYPE: Article

RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Immunohistochemical and/or indirect immunofluorescence analysis with monoclonal antibody (MAb) H19 demonstrated the expression of protectin (CD59) in 54 surgically removed metastatic melanoma lesions and on 8 out of 12 melanoma cell lines. CD59 expression had a low degree of intra- and intertumor heterogeneity. SDS-PAGE analysis showed that the molecular weight of CD59 expressed on melanoma cells is about 20 kDa. Treatment of melanoma cells with 5 U/ml of phosphatidylinositol-specific phospholipase C completely abolished cell-surface expression of CD59. Interferon-gamma and/or tumor necrosis factor-alpha or phorbol 12-myristate 13-acetate neither modulated the expression of CD59 by melanoma cells nor influenced the amounts of CD59-specific mRNA. F(ab')-2 fragments of anti-CD59 MAb YTH53.1 did not inhibit the lysis of melanoma cells by allogeneic natural killer (NK) cells or lymphokine-activated killer (LAK) cells. In contrast, the whole Ig molecule of MAb H 19 or YTH53.1 significantly (p lt 0.05) enhanced NK-cell-mediated lysis of melanoma cells, suggesting the induction of antibody-dependent cell-mediated cytotoxicity. Lastly, masking of CD59 by MAb YTH53.1 or its F(ab')-2 fragments significantly (p lt 0.05) enhanced, in a dose-dependent fashion, the lysis of anti-GD3-sensitized melanoma cells by homologous complement. These data demonstrate that CD59 expressed by human melanoma cells might regulate host-tumor interaction by protecting neoplastic cells from complement-mediated lysis.

---------- prtectin cd59 ----------

13/7/5 (Item 5 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0009627241 BIOSIS NO.: 199598095074
Expression and function of the complement membrane attack complex inhibitor protectin (CD59) on human breast cancer cells
AUTHOR: **Hakulinen Juha; Meri Seppo (Reprint)**
AUTHOR ADDRESS: Dep. Bacteriol. Immunol., P.O. Box 21, Univ. Helsinki, FIN-00014 Helsinki, Finland**Finland
JOURNAL: **Laboratory Investigation 71 (6): p820-827 1994 1994**
ISSN: 0023-6837
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: BACKGROUND: Normal human cells resist the lytic activity of homologous complement (C) by expressing inhibitory molecules on their cell membranes. Recently, it has become increasingly evident that information on C inhibitors on malignant tumor cells is crucial before considering any immunotherapeutic attempts with C-activating antibodies. As one of the most potent inhibitors of C lysis is protectin (CD59), we have examined its expression and function on human breast cancer cells. EXPERIMENTAL DESIGN: Immunofluorescence microscopy was used to detect protectin expression on solid breast tumor samples (N = 12). Using immunoaffinity chromatography, protectin was isolated from the membranes of cultured MCF7 and T47D breast cancer cells. The purified proteins were incorporated into heterologous cells to study their C inhibitory activities. The reactivity of tumor cell protecting with terminal C complexes was examined by sucrose density ultracentrifugation analysis. A chromium release assay was used to study the effects of protectin neutralization on the

sensitivity of MCF7 and T47D cells to C-mediated cytotoxicity. RESULTS: Protectin was found to be strongly expressed by all human breast cancer tumors examined. The affinity-purified protecting had a glycophosphoinositollipid anchor and migrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis as glycosylated smears of 19 to 25 kilodaltons. Protectin isolated from T47D cells bound to nascent C5b-9 complexes generated in human sera and inhibited C lysis of guinea pig erythrocytes when incorporated into their cell membranes. C-mediated killing of breast cancer cells could be significantly enhanced after treatment of the cells with F(ab')-2 fragments of the anti-protectin monoclonal antibody YTH53.1. CONCLUSIONS: Human breast cancer cells resist C membrane attack by expressing protectin on their cell membranes. Neutralization of protectin on the surface of the tumor cells increases their sensitivity to C lysis.

---------- cd59 protectin ----------
---------- cd59 ----------

13/7/9 (Item 9 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0009026551 BIOSIS NO.: 199497047836
Expression of CD59, a complement regulator protein and a second ligand of the CD2 molecule, and CD46 in normal and neoplastic colorectal epithelium
AUTHOR: **Koretz K (Reprint); Bruederlein S; Henne C; Moeller P**
AUTHOR ADDRESS: Inst. Pathology, Univ. Heidelberg, Im Neuenheimer Feld 220, 69120 Heidelberg, Germany**Germany
JOURNAL: **British Journal of Cancer 68 (5): p926-931 1993 1993**
ISSN: 0007-0920
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: CD59 (protectin) and CD46 (membrane cofactor protein, MCP) are membrane-bound complement regulator proteins which inhibit complement-mediated cytolysis of autologous cells. CD59, a phosphatidyl-inositol-anchored glycoprotein, inhibits the formation of the terminal membrane attack complex (MAC) of complement and was found to be a second ligand for CD2 contributing to T-cell activation. In 20 colorectal normal mucosa samples, in ten adenomas, 71 carcinomas and in ten liver metastases derived thereof, CD59 was inconsistently expressed in the epithelial compartment. In carcinomas CD59 expression in the whole neoplastic compartment was more often found in well- and moderately differentiated tumours. By contrast, focal expression or even complete lack of CD59 was more often found in poorly differentiated tumours (P = 0.021). In addition, carcinomas without metastases at the time of operation (Dukes A/B) more often expressed CD59 in the entire neoplastic population compared to those carcinomas which had already metastasised (P = 0.018). There was no correlation between the mode of CD59 expression in colorectal carcinomas and the tumour type or location. CD46 has C3b/C4b binding and factor-I dependent cofactor activity and is broadly expressed in various cells and tissues. In the epithelial compartment of normal colorectal mucosa, of all adenomas, carcinomas and their liver metastases, CD46 was expressed throughout the epithelial compartment. Since CD46 was consistently expressed in colorectal carcinomas the low expression or even lack of CD59 in a subset of tumours might not lead to critical complement-mediated attack of CD59-negative tumour cells. Regarding CD59 as a natural T-cell ligand involved in cognate T-cell - target-cell interaction, however,

loss of CD59 might well be a selection advantage, provided that tumour antigen-mediated T-cell toxicity in colorectal carcinoma exists.

---------- cd59 protectin -

13/7/10 (Item 10 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0009026189 BIOSIS NO.: 199497047474
Modulation of protectin (CD59 antigen) cell surface expression on human neoplastic cell lines
AUTHOR: Sedlak J; Hunakova L; Duraj J; Grofova M; Chorvath B
AUTHOR ADDRESS: Cancer Res. Inst., Slovak Acad. Sci., 812 32 Bratislava, Slovakia**Slovakia
JOURNAL: Neoplasma (Bratislava) 40 (6): p337-340 1993 1993
ISSN: 0028-2685
DOCUMENT TYPE: Article
RECORD TYPE: Citation
LANGUAGE: English

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Sent: Sunday, April 10, 2005 2:07 PM
To: STIC-ILL
Subject: boussiotis 09 / 995,519 cd59

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---------- cd59 ----------

9/7/4 (Item 4 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0012614997 BIOSIS NO.: 200000333310
CD59 expressed on a tumor cell surface modulates decay-accelerating factor expression and enhances tumor growth in a rat model of human neuroblastoma
AUTHOR: **Chen Shaohua; Caragine Theresa; Cheung Nai-Kong V; Tomlinson Stephen (Reprint)**
AUTHOR ADDRESS: Department of Microbiology and Immunology, Medical University of South Carolina, BSB 201, 173 Ashley Avenue, Charleston, SC, 29425, USA**USA
JOURNAL: **Cancer Research 60 (11): p3013-3018 June 1, 2000 2000**
MEDIUM: print
ISSN: 0008-5472
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: It has been hypothesized that complement inhibitors expressed on the surface of tumor cells prevent effective immune-mediated clearance. Whereas there are in vitro data to support this hypothesis, the species-selective activity of complement inhibitors has been a hindrance to investigating the role of membrane-bound complement inhibitors in rodent models of human cancer. The CD59-positive LAN-1 human neuroblastoma cell line was significantly more sensitive to lysis by rat complement than by human complement, illustrating the species selectivity of endogenously expressed complement inhibitors. Transfection of LAN-1 cells with rat CD59, an inhibitor of the terminal cytolytic membrane attack complex, effectively protected the cells from lysis by rat complement in vitro. When LAN-1 cells stably expressing rat CD59 were inoculated into immune-deficient rats, the onset of tumor growth and the rate of tumor growth were significantly enhanced compared with those of control-transfected LAN-1 cells. These data show directly that the expression of a complement inhibitor on a tumor cell promotes tumor growth. Flow cytometric analysis revealed that the endogenous expression of decay-accelerating factor (DAF), an inhibitor of complement activation, was up-regulated on the

surface of cells after in vivo growth. Of further interest, higher levels of DAF were present on CD59-transfected cells than on control-transfected cells derived from tumors. Increased DAF expression correlated with decreased complement deposition on the tumor cell surface. These results show that expression of complement inhibitors on a tumor cell has functional consequences with regard to complement deposition in vivo and indicate that CD59 can indirectly effect complement activation and C3 deposition in vivo via a link between CD59 and DAF expression.

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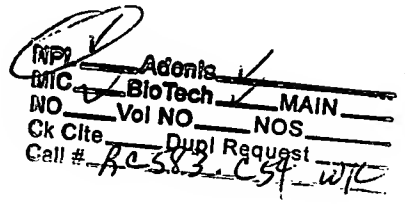

---------- cd59 ----------

9/7/5 (Item 5 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
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0011828122 BIOSIS NO.: 199900087782
Protection of human breast cancer cells from complement-mediated lysis by expression of heterologous CD59
AUTHOR: **Yu J; Caragine T; Chen S; Morgan B P; Frey A B; Tomlinson Stephen (Reprint)**
AUTHOR ADDRESS: New York Univ. Med. Cent., Dep. Pathol., MSB 127, 550 First Avenue, New York, NY 10016, USA**USA
JOURNAL: **Clinical and Experimental Immunology 115 (1): p13-18 Jan., 1999 1999**
MEDIUM: print
ISSN: 0009-9104
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: CD59, decay accelerating factor (DAF) and membrane cofactor protein (MCP) are widely expressed cell surface glycoproteins that protect host cells from the effects of homologous complement attack. Complement inhibitory activity of these proteins is species-selective. We show that the human breast cancer cell line MCF7 is relatively resistant to lysis by human complement, but is effectively lysed by rat or mouse complement. CD59, DAF and MCP were all shown to be expressed by MCF7. The species-selective nature of CD59 activity was used to demonstrate directly the effectiveness of CD59 at protecting cancer cells from complement-mediated lysis. cDNAs encoding rat and mouse CD59 were separately transfected into MCF7 cells, and cell populations expressing high levels of the rodent CD59 were isolated by cell sorting. Data show that rat and mouse CD59 were highly effective at protecting transfected MCF7 cells from lysis by rat and mouse complement, respectively. Data further reveal that rat CD59 is not effective against mouse complement, whereas mouse CD59 is effective against both mouse and rat complement. These studies establish a model system for relevant in vivo studies aimed at determining the effect of complement regulation on tumorigenesis, and show that for effective immunotherapy using complement-activating anti-tumour antibodies, the neutralization of CD59 and/or other complement inhibitory molecules will probably be required.

---------- cd59 ----------

9/7/7 (Item 2 from file: 73)
DIALOG(R)File 73:EMBASE
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12209865 EMBASE No: 2003322989
Obstacles to cancer immunotherapy: Expression of membrane complement regulatory proteins (mCRPs) in tumors

Fishelson Z.; Donin N.; Zell S.; Schultz S.; Kirschfink M.
Z. Fishelson, Dept. of Cell/Developmental Biology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978 Israel
AUTHOR EMAIL: lifish@post.tau.ac.il
Molecular Immunology (MOL. IMMUNOL.) (United Kingdom) 2003, 40/2 (109-123)
CODEN: IMCHA ISSN: 0161-5890
DOCUMENT TYPE: Journal ; Conference Paper
LANGUAGE: ENGLISH SUMMARY LANGUAGE: ENGLISH
NUMBER OF REFERENCES: 209

Monoclonal antibodies (mAbs) are being increasingly used in cancer therapy owing to their ability to recognize specifically cancer cells and to activate complement- and cell-mediated cytotoxicity and/or to induce growth arrest or apoptosis. The therapeutic potential of anticancer antibodies is significantly limited due to the ability of cancer cells to block killing by complement. Of the multiple resistance strategies exploited by cancer cells, the expression of membrane complement regulatory proteins (mCRPs), such as CD46 (membrane cofactor protein (MCP)), CD55 (decay-accelerating factor (DAF)), CD35 (complement receptor type-1 (CR1)) and CD59, has received most attention. CD46, CD55 and CD35 block the complement cascade at the C3 activation stage and CD59 prevents assembly of the membrane attack complex of complement (MAC). These proteins protect normal tissues from accidental injury by activated complement, but also confer resistance on cancer cells, thereby limiting the effect of complement-fixing monoclonal antibodies. Expression of mCRPs on malignant cells is highly variable, yet there is clear indication that certain tumors express higher mCRP levels than the normal tissue from which they have evolved. mCRP level of expression and cellular location may also vary during malignant transformation and between differentiated and undifferentiated tumors. Neutralizing anti-mCRP mAbs have been used in vitro to elucidate the significance of mCRP expression to the tumor complement resistance phenotype. In general, CD59 appears to be the most effective mCRP protecting tumor cells from complement-mediated lysis. Nevertheless, it acts additively, and in certain tumors even synergistically, with CD55 and CD46. It is envisaged that treatment of cancer patients with mCRP blocking antibodies targeted specifically to cancer cells in combination with anticancer complement-fixing antibodies will improve the therapeutic efficacy.

--------- protectin -------

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13/7/4 (Item 4 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0009863458 BIOSIS NO.: 199598331291
Expression of protectin (CD59) in human melanoma and its functional role in cell- and complement-mediated cytotoxicity
AUTHOR: **Brasoveanu Lorelei I; Altomonte Maresa; Gloghini Annunziata; Fonsatti Ester; Coral Sandra; Gasparollo Aldo; Montagner Roberto; Cattarossi Ilaria; Simonelli Cecilia; Cattelan Alessandro; Attadia Vincenza; Carbone Antonino; Maio Michele (Reprint)**
AUTHOR ADDRESS: Advanced Immunotherapy Unit, INRCCS-CRO, Via Pedemontana Occ.le 12, Aviano, Italy**Italy
JOURNAL: **International Journal of Cancer 61 (4): p548-556 1995 1995**
ISSN: 0020-7136
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

Phillip Gambel
1644 4/11
09/995,519

ABSTRACT: Immunohistochemical and/or indirect immunofluorescence analysis with monoclonal antibody (MAb) H19 demonstrated the expression of protectin (CD59) in 54 surgically removed metastatic melanoma lesions and on 8 out of 12 melanoma cell lines. CD59 expression had a low degree of intra- and intertumor heterogeneity. SDS-PAGE analysis showed that the molecular weight of CD59 expressed on melanoma cells is about 20 kDa. Treatment of melanoma cells with 5 U/ml of phosphatidylinositol-specific phospholipase C completely abolished cell-surface expression of CD59. Interferon-gamma and/or tumor necrosis factor-alpha or phorbol 12-myristate 13-acetate neither modulated the expression of CD59 by melanoma cells nor influenced the amounts of CD59-specific mRNA. F(ab')-2 fragments of anti-CD59 MAb YTH53.1 did not inhibit the lysis of melanoma cells by allogeneic natural killer (NK) cells or lymphokine-activated killer (LAK) cells. In contrast, the whole Ig molecule of MAb H 19 or YTH53.1 significantly (p lt 0.05) enhanced NK-cell-mediated lysis of melanoma cells, suggesting the induction of antibody-dependent cell-mediated cytotoxicity. Lastly, masking of CD59 by MAb YTH53.1 or its F(ab')-2 fragments significantly (p lt 0.05) enhanced, in a dose-dependent fashion, the lysis of anti-GD3-sensitized melanoma cells by homologous complement. These data demonstrate that CD59 expressed by human melanoma cells might regulate host-tumor interaction by protecting neoplastic cells from complement-mediated lysis.

---------- prtectin cd59 ----------

13/7/5 (Item 5 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0009627241 BIOSIS NO.: 199598095074
Expression and function of the complement membrane attack complex inhibitor protectin (CD59) on human breast cancer cells
AUTHOR: **Hakulinen Juha; Meri Seppo (Reprint)**
AUTHOR ADDRESS: Dep. Bacteriol. Immunol., P.O. Box 21, Univ. Helsinki, FIN-00014 Helsinki, Finland**Finland
JOURNAL: **Laboratory Investigation 71 (6): p820-827 1994 1994**
ISSN: 0023-6837
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: BACKGROUND: Normal human cells resist the lytic activity of homologous complement (C) by expressing inhibitory molecules on their cell membranes. Recently, it has become increasingly evident that information on C inhibitors on malignant tumor cells is crucial before considering any immunotherapeutic attempts with C-activating antibodies. As one of the most potent inhibitors of C lysis is protectin (CD59), we have examined its expression and function on human breast cancer cells. EXPERIMENTAL DESIGN: Immunofluorescence microscopy was used to detect protectin expression on solid breast tumor samples (N = 12). Using immunoaffinity chromatography, protectin was isolated from the membranes of cultured MCF7 and T47D breast cancer cells. The purified proteins were incorporated into heterologous cells to study their C inhibitory activities. The reactivity of tumor cell protecting with terminal C complexes was examined by sucrose density ultracentrifugation analysis. A chromium release assay was used to study the effects of protectin neutralization on the sensitivity of MCF7 and T47D cells to C-mediated cytotoxicity. RESULTS: Protectin was found to be strongly expressed by all human breast cancer tumors examined. The affinity-purified protecting had a glycophosphoinositollipid anchor and migrated in sodium dodecyl

Fishelson Z.; Donin N.; Zell S.; Schultz S.; Kirschfink M.
Z. Fishelson, Dept. of Cell/Developmental Biology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978 **Israel**
AUTHOR EMAIL: lifish@post.tau.ac.il
Molecular Immunology (MOL. IMMUNOL.) (United Kingdom) 2003, 40/2 (109-123)
CODEN: IMCHA ISSN: 0161-5890
DOCUMENT TYPE: Journal ; Conference Paper
LANGUAGE: ENGLISH SUMMARY LANGUAGE: ENGLISH
NUMBER OF REFERENCES: 209

Phillip Damiel
1644 4/11
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--------- protectin ----

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13/7/4 (Item 4 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

0009863458 BIOSIS NO.: 199598331291
Expression of protectin (CD59) in human melanoma and its functional role in cell- and complement-mediated cytotoxicity
AUTHOR: **Brasoveanu Lorelei I; Altomonte Maresa; Gloghini Annunziata; Fonsatti Ester; Coral Sandra; Gasparollo Aldo; Montagner Roberto; Cattarossi Ilaria; Simonelli Cecilia; Cattelan Alessandro; Attadia Vincenza; Carbone Antonino; Maio Michele (Reprint)**
AUTHOR ADDRESS: Advanced Immunotherapy Unit, INRCCS-CRO, Via Pedemontana Occ.le 12, Aviano, Italy**Italy
JOURNAL: **International Journal of Cancer 61 (4): p548-556 1995 1995**
ISSN: 0020-7136
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

0023-6837/94/7106-0820$03.00/0
LABORATORY INVESTIGATION

Vol. 71, No. 6, p. 820, 1994
Printed in U.S.A.

Expression and Function of the Complement Membrane Attack Complex Inhibitor Protectin (CD59) on Human Breast Cancer Cells

JUHA HAKULINEN AND SEPPO MERI

Department of Bacteriology and Immunology, FIN-00014 University of Helsinki, Helsinki, Finland

BACKGROUND: Normal human cells resist the lytic activity of homologous complement (C) by expressing inhibitory molecules on their cell membranes. Recently, it has become increasingly evident that information on C inhibitors on malignant tumor cells is crucial before considering any immunotherapeutic attempts with C-activating antibodies. As one of the most potent inhibitors of C lysis is protectin (CD59), we have examined its expression and function on human breast cancer cells.

EXPERIMENTAL DESIGN: Immunofluorescence microscopy was used to detect protectin expression on solid breast tumor samples ($N = 12$). Using immunoaffinity chromatography, protectin was isolated from the membranes of cultured MCF7 and T47D breast cancer cells. The purified proteins were incorporated into heterologous cells to study their C inhibitory activities. The reactivity of tumor cell protectins with terminal C complexes was examined by sucrose density ultracentrifugation analysis. A chromium release assay was used to study the effects of protectin neutralization on the sensitivity of MCF7 and T47D cells to C-mediated cytotoxicity.

RESULTS: Protectin was found to be strongly expressed by all human breast cancer tumors examined. The affinity-purified protectins had a glycophosphoinositollipid anchor and migrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis as glycosylated smears of 19 to 25 kilodaltons. Protectin isolated from T47D cells bound to nascent C5b-9 complexes generated in human sera and inhibited C lysis of guinea pig erythrocytes when incorporated into their cell membranes. C-mediated killing of breast cancer cells could be significantly enhanced after treatment of the cells with $F(ab')_2$ fragments of the anti-protectin monoclonal antibody YTH53.1.

CONCLUSIONS: Human breast cancer cells resist C membrane attack by expressing protectin on their cell membranes. Neutralization of protectin on the surface of the tumor cells increases their sensitivity to C lysis.

Additional key words: HER-2, *neu*, Immunotherapy, Decay accelerating factor, Glycophosphoinositol.

The lytic activity of human complement (C) against homologous cells is regulated by several inhibitor molecules on cell membranes (1). The key enzymes of the C cascade, C3/C5 convertases, are inhibited by C3b receptor, CR1 (CD35) (2), DAF (CD55) (3) and MCP (CD46) (4) that dissociate the convertase complexes (CR1, DAF) or promote proteolytic inactivation of their noncatalytic subunits C4b or C3b (CR1, MCP). Two inhibitors of the membrane attack complex (MAC) have been described: a 65 kilodalton (kd) protein, that has been called a C8-binding protein (5), homologous restriction factor (6), or MAC-inhibiting protein (6) and CD59, that was isolated first from human erythrocyte membranes (7, 8) and has been referred to as MACIF (9) HRF20 (10), MIRL (11), or protectin (12, 13). Protectin is a glycophosphoinositol-anchored membrane glycoprotein that in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), has an apparent molecular weight of 18 to 20 kd (7, 8). Mass spectrometric analysis has shown an average molecular mass of approximately 13 kd for soluble phospholipid-free protectin (S. Meri, T. Lehto, C. W. Sutton, J. Tyynelä, and M. Baumann, manuscript submitted for publication). Protectin inhibits formation of the MAC by preventing C5b-8 catalyzed insertion of C9 into lipid bilayers (12, 14). In human tissues, protectin has been shown to be expressed by endothelial and epithelial cells of several organs (15). It has also been detected on cultured endothelial cells (16, 17), peripheral blood mononuclear cells (18), glomerular epithelial cells (19), and spermatozoa (20). In addition to protection provided by the membrane inhibitors, nucleated cells can resist C attack by eliminating MAC-complexes via exo- and endocytosis (21).

The possible utilization of the cytotoxic C system

against human cancer cells requires that the tumor cells be recognized by complement-activating tumor-specific monoclonal antibodies (mAb) and that the tumor cell resistance against homologous C can be overcome. Certain types of tumor cells express tumor-associated or tumor-specific antigens that may be discriminated from normal cell surface antigens by specific antibodies (reviewed *e.g.*, in reference 22) The restricted distribution and tumor growth-promoting activity of cellular receptors for certain hormones and growth factors make them potential targets for immunotherapy with mAb. One such candidate receptor is the *neu/C-erbB-2/HER2* oncoprotein that is overexpressed by malignant and metastatic breast cancer cells (23).

Tumor cells do not usually elicit a significant cytotoxic humoral immune response against them. As evidence for a possible *in vivo* C attack against tumor cells Niculescu *et al.* (24) have shown deposition of C5b-9 complexes on breast carcinoma cell membranes. In our own experience (Hovi *et al.*, unpublished observations) complement membrane attack against tumor cells can occasionally be detected *in vivo* but this seems to be a consequence of tissue ischemia and necrosis rather than a reflection of a genuine immune response. However, this does not exclude the possibility that complement attack against tumors could be recruited with tumor-specific monoclonal antibodies. Thus far, the role of membrane inhibitors of the complement membrane attack complex in protecting human tumor cells against lysis by homologous complement has largely been unexplored, although it certainly is one of the critical factors when mAb-mediated anti-tumor immunotherapy is being considered. Our goals in the present study were to investigate whether protectin is expressed on human breast cancer cells and whether the tumor cell protectin restricts complement mediated lysis of the malignant cells. It was found that protectin was strongly expressed by all solid breast tumor cases examined. The tumor cell protectins exhibited similar structural and functional properties as protectins isolated from other cell types. Neutralization of the breast tumor cell protectin with specific monoclonal anti-protectin antibodies led to significantly enhanced killing of cultured tumor cells. Targeted sensitization of tumor cells to homologous C attack may offer new possibilities for the immunotherapy of human breast cancer.

EXPERIMENTAL DESIGN

The expression of protectin was examined on breast tumor samples *in vivo* and on breast cancer cell lines (MCF7 and T47D) *in vitro* using immunofluorescence (IF) microscopy and specific monoclonal anti-protectin antibodies (YTH53.1, BRIC 229). Protectin was purified from detergent solubilized cell membranes of cultured MCF7 and T47D cells by YTH53.1-Sepharose immunoaffinity chromatography. The isolated proteins were analyzed by SDS-PAGE and immunoblotting with YTH53.1 and BRIC 229 mAb. To study the complement inhibitory activity, various amounts of the purified proteins were incorporated into guinea pig erythrocytes (GPE) that were treated with normal human serum (NHS). Reactivity of the tumor cell protectins with terminal complement complexes (TCC) was examined by incubating radiolabeled protectin from T47D cells with inulin activated NHS and separating the protectin-C5b-9 complexes by sucrose density gradient ultracentrifugation. In complement-mediated cytotoxicity tests MCF7 cells were labeled with ^{51}chromium. Protectin on the cell membranes was neutralized with various amounts of $F(ab')_2$ fragments of the YTH53.1 mAb. After sensitizing the cells with polyclonal anti-MCF7 antibodies the cells were exposed to NHS and cell lysis determined from radioactivity released.

RESULTS AND DISCUSSION

Immunofluorescence Microscopy

Cultured MCF7 and T47D cells were found to express protectin on their cell membranes as demonstrated by IF microscopy in Figure 1. Cells stained positive for protectin by both the YTH53.1 and BRIC 229 mAb. Slight enhancement of staining was observed at sites of cell contacts. A similar pattern was observed with fluorescein isothiocyanate-labeled $F(ab')_2$ fragments of YTH53.1. No reactivity was seen with a control rat IgG2b mAb against glycophorin A (YTH89.1) or with the fluorescein conjugates alone. Examination of MCF7 and T47D cells with the GB24 mAb showed that both cell types expressed membrane cofactor protein (MCP, CD46). Expression of decay accelerating factor (DAF, CD55) was stronger on MCF7 (Fig. 1*e*) than on T47D cells (not shown).

To study the expression of protectin in human breast tumors *in vivo* a total of 12 specimens (ductal carcinoma: 5 cases, metastases of ductal carcinoma: 2 cases, lobular carcinoma: 3 cases, and fibroadenoma of the breast: 2 cases) were examined by IF microscopy. Protectin was found to be strongly expressed by all the tumors, of which examples are shown in Figure 2. Staining appeared significantly stronger in the tumor cells than in the neighboring connective tissue. In cells that showed an epithelial polarized pattern, the strongest staining for protectin was seen at the apical membranes. A very strong staining was seen within the ducts and on cell surfaces adjacent to the lumen (Fig. 2*a*). In all tissues, the endothelia of blood vessels appeared strongly positive for protectin. In general, the membrane staining pattern was identical with the YTH53.1 and BRIC 229 mAb. In some tumors, however, the YTH53.1 mAb and its $F(ab')_2$ fragments showed an additional staining of intracellular filaments in acetone-treated tissue sections (not shown). No similar staining was seen with the BRIC 229 mAb (or with the YTH89.1 mAb with the same isotype as YTH53.1) suggesting that the reactivity of the YTH53.1 mAb with the filaments was due to a Fab-region-mediated nonspecific reactivity and was therefore not considered as specific for protectin. Although both mAbs are capable of blocking the functional activity of protectin, this observation suggests these mAbs may recognize slightly different conformational epitopes on protectin.

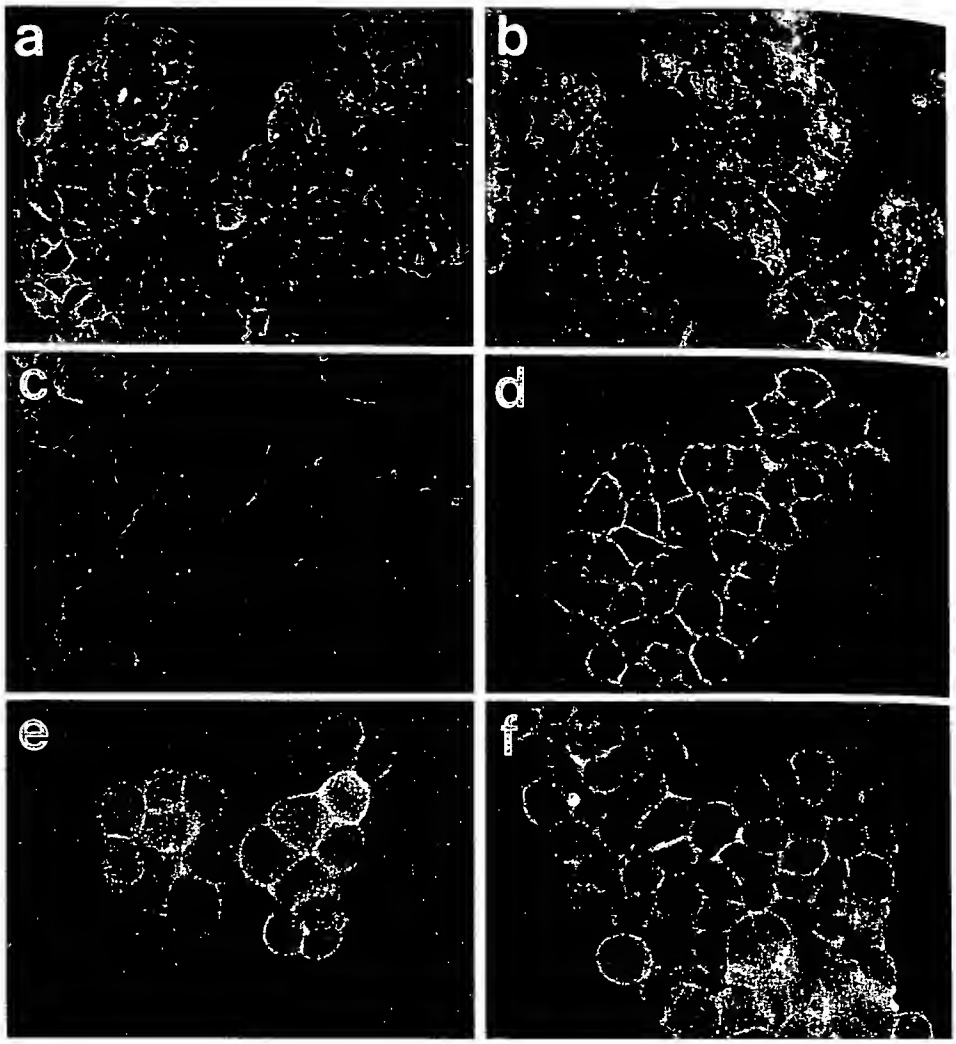

FIG. 1. Demonstration of protectin on cultured MCF7 and T47D breast cancer cells by indirect immunofluorescence microscopy. MCF7 (*a*, *c-f*) or T47D (*b*) cells grown on coverslips (*a*-c) or harvested into suspension (*d-f*) were incubated with the BRIC 229 (*a* and *b*) or YTH53.1 (*d*) anti-protectin mAb or with antibodies against glycophorin A (YTH89.1; *c*, decay accelerating factor (BRIC 216; *e*, or membrane cofactor protein (GB24; *f*, After treatment with the respective fluorescein isothiocyanate-conjugated anti-rat or anti-mouse antibodies the cells were fixed with 3% formaldehyde. Controls without the primary antibody showed no fluorescence.

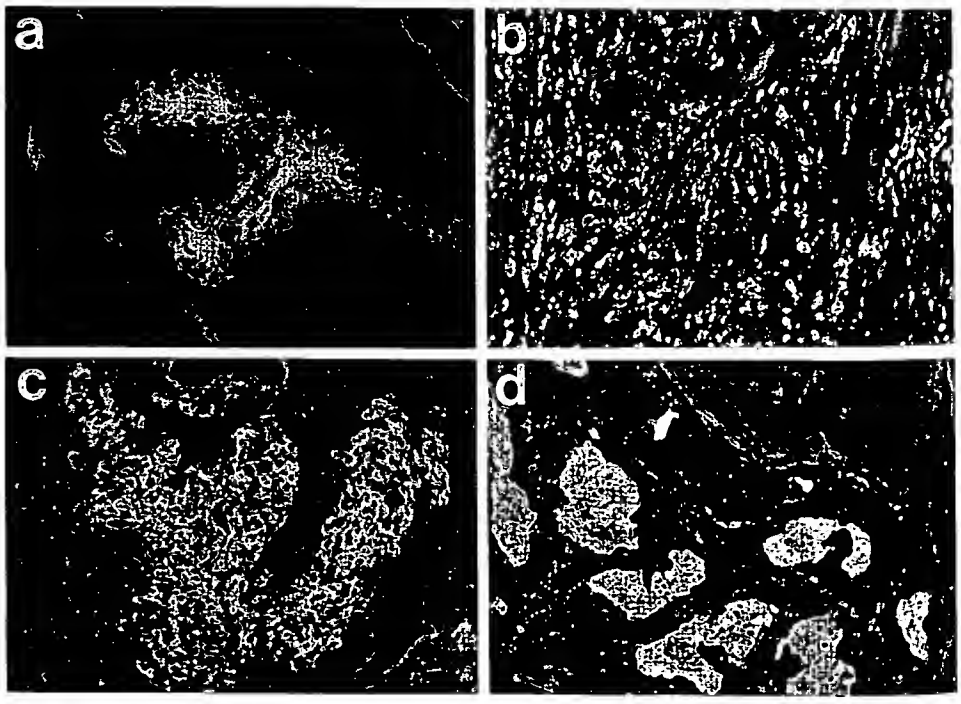

FIG. 2. Immunofluorescence detection of protectin on solid human breast cancer tumors. Cryostat sections from a fibroadenoma of the breast (*a*), an infiltrating lobular carcinoma (*b*), invasive ductal carcinoma (*c*), and ductal carcinoma *in situ* (*d*), were fixed with acetone and stained for protectin with the YTH53.1 mAb.

Purification and Characterization of Protectin from MCF7 and T47D Cell Lines

As it was evident that breast cancer cells strongly expressed protectin, the molecular characteristics of protectin in the MCF7 and T47D cell lines were examined. The MCF7 and T47D cell membrane-extracts were solubilized with 60 mM n-octyl-β-D-glucopyranoside. The soluble extracts were subjected to YTH53.1 affinity column and the eluted material was analyzed by SDS-PAGE and immunoblotting. As shown in Figure 3, the isolated proteins migrated as broad bands with apparent molecular weights ranging from 19 to 25 kd. The pattern was similar to that of protectin purified from erythrocyte cell membranes although, depending on the amount of sample loaded in the gel, the smears of erythrocyte protectin sometimes extended to 30 kd (not shown). The smear for soluble protectin isolated from human urine started from a slightly higher apparent molecular weight (21 kd) than those of the lipid-anchored protectins (Fig. 3). Immunoreactivity of the tumor cell protectin with the YTH53.1 or BRIC 229 mAb was abolished if the antigens were treated with a reducing agent, such as β-mercaptoethanol, before SDS-PAGE. Additional 28 and 32 kd bands in the immunoblots are apparently due to nonspecific reactivity of the samples with the secondary antibodies, because these bands were seen also in the controls where the primary antibodies were omitted. Affinity-purified protectin did not become stained by the enzyme-conjugate alone.

Complement-Mediated Killing of MCF7 and T47D Cells

Cytotoxic effect of human complement against MCF7 and T47D cells was examined in the presence and absence of protectin-neutralizing antibodies. When MCF7 cells were treated with $F(ab')_2$ fragments of the YTH53.1 mAb an increase in antibody-induced complement-mediated killing of the cells was observed (Fig. 4). Approximately 12 μg/ml of the antibody was required to obtain full neutralization of the tumor-cell protectin activity. To sensitize T47D and MCF7 cells to complement lysis, a polyclonal rabbit antibody S2 was found most effective. A mAb (clone 528) against HER2/c-erbB-2 was ineffective in this respect. In the absence of the sensitizing rabbit antibodies, no lysis occurred with the YTH53.1 $F(ab')_2$ alone. Also, in separate experiments, the $F(ab')_2$ fragments did not induce lysis of human erythrocytes by human complement, whereas the whole parent antibody, apparently by activating the classical complement pathway, lysed human erythrocytes in the presence of complement.

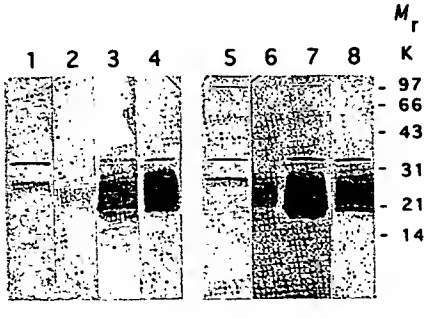

FIG. 3. Immunoblotting analysis of protectin from MCF7 and T47D cells. Samples of affinity-purified protectin from urine (*lanes 2 and 6*), T47D (*lanes 3 and 7*), and MCF7 (*lanes 4 and 8*) cells were run on a 15% SDS-PAGE slab gel under nonreducing conditions, transferred to nitrocellulose and immunostained using YTH53.1 rat (*lanes 2–4*) or R2 rabbit anti-protectin antibodies (*lanes 6–8*). Bound antibodies were detected by using alkaline phosphatase-conjugated secondary antibodies. *Lanes 1 and 5* represent control (protectin from MCF7 cells) where no primary antibody was added.

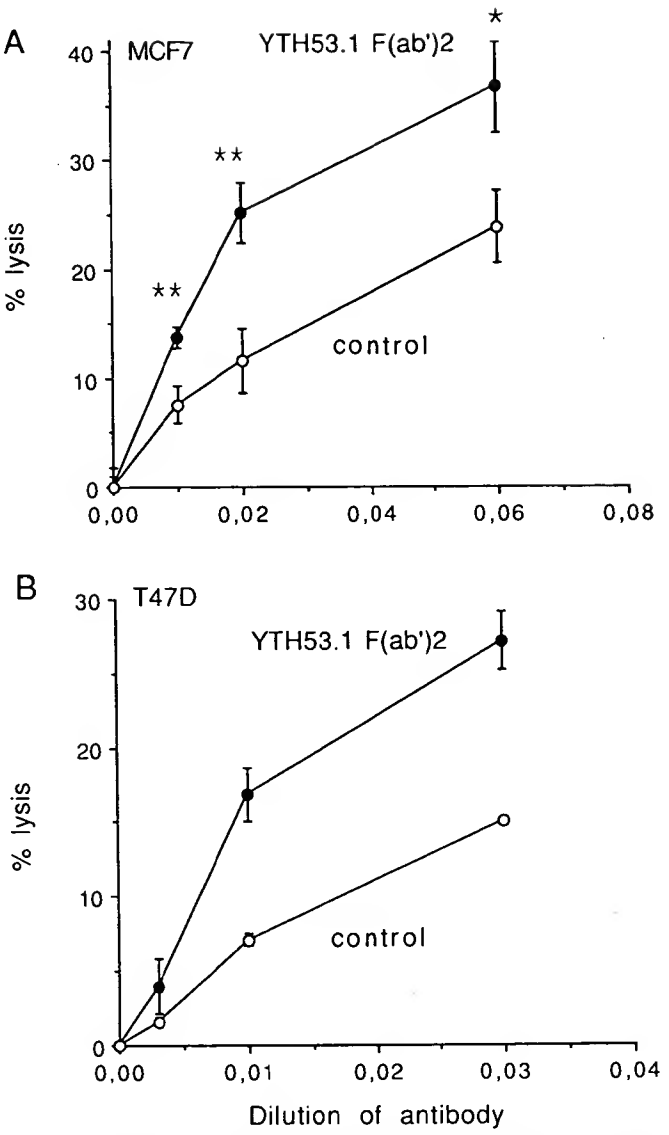

FIG. 4. Enhancement of complement lysis of MCF7 (*A*) and T47D (*B*) cells by $F(ab')_2$ fragments of the YTH53.1 mAb. ^{51}Cr-labeled cells (8×10^5/ml) were sensitized with different dilutions of polyclonal anti-MCF7 serum in the presence (10 μg/ml) or absence of $F(ab')_2$ fragments of YTH53.1 and exposed to normal human serum (1/4 dilution). Cell lysis was quantified as release of ^{51}Cr into the medium. Mean ± SEM values (*A*, $N = 4$; *B*, $N = 2$) are shown. *, $p < 0.05$; **, $p < 0.01$; paired Student's *t*-test.

Functional Activity of Protectin Purified from T47D Cells

When purified radiolabeled T47D-protectin (11 ng) was mixed with rabbit erythrocytes (2×10^7 cells) in the

presence of 60 μM n-octyl-β-D glucopyranoside 12% of the protein became incorporated into the cells indicating that the isolated T47D-protectin had not lost its glycophospholipid anchor. Under similar conditions, only 0.4% of ^{125}I-labeled urinary protectin became associated with the erythrocytes. Incorporation of T47D-protectin into GPE was found to lead to inhibition of lysis of the cells by NHS (Fig. 5). The functional activity of T47D-protectin was equivalent to that of protectin purified from erythrocyte membranes (12). The C-lysis inhibitory effect of incorporated T47D-protectin could be blocked by F(ab')$_2$ fragments of the YTH53.1 mAb. When mixed with NHS, the ^{125}I-labeled T47D-protectin bound to the soluble SC5b-9 complex during activation of the serum with 4% inulin (Fig. 6). No similar association occurred during activation of guinea pig serum.

Discussion

In the present study, the complement membrane attack complex inhibitor, protectin was found to be expressed and functionally active on human breast cancer cells. Immunofluorescence studies with anti-protectin mAb showed that the molecule was located on the membranes of both cultured breast cancer cells (MCF7, T47D) and in solid breast cancer tumors examined on cryostat sections. Purified protectin from T47D cells became inserted into heterologous erythrocytes with the help of its glycophosphoinositol-lipid anchor and protected the cells against complement lysis. The purified tumor cell protectin had properties similar to erythrocyte protectin. In Western blots, the proteins were visible as 19 to 25 kd broad smears reacting with the YTH53.1 and BRIC 229 monoclonal antibodies. Heterogeneity in the apparent molecular weight of protectin is due to variable branching and sialylation of the *N*-linked oligosaccharide side chain, which is linked to the Asn^{18} residue in the polypeptide chain and constitutes about 25% of the molecular mass of protectin (13).

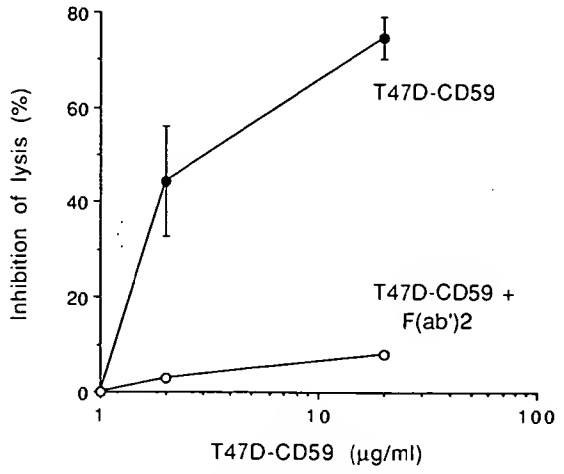

FIG. 5. Inhibition of complement lysis of guinea pig erythrocytes by T47D-protectin incorporated into cell membranes. GPE were incubated with different amounts of T47D-protectin and exposed to normal human serum (diluted 1/24) with (10 μg/ml) or without YTH53.1 F(ab')$_2$ fragments. Lysis was measured as release of hemoglobin at A_{412}. Mean ± SEM values from two independent experiments are shown.

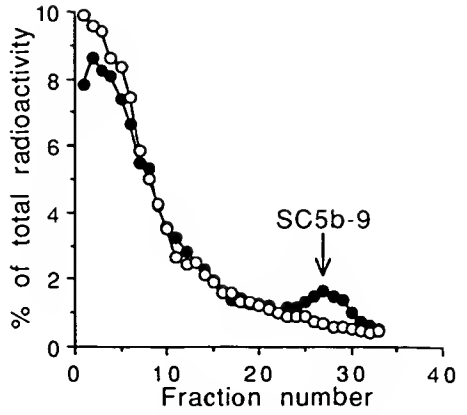

FIG. 6. Binding of ^{125}I-labeled T47D-protectin to terminal complement complexes. NHS (60 μl) was activated with 4% inulin in the presence of ^{125}I-labeled T47D-protectin (11 ng; ●). Control (○) was treated similarly except that no inulin was added. SC5b-9 bound radioactivity was separated from free ligand by sucrose density gradient (10 to 50%) ultracentrifugation.

The affinity-purified T47D-protectin retained its functional activity as judged by inhibition of complement lysis of GPE when introduced into the cells. GPE activate complement via the alternative pathway leading to deposition of MAC on the cell membranes and eventually to cell lysis. Protectin inhibits lysis by binding to the nascent C5b-8-complex and preventing the insertion and polymerization of C9 (12, 14, 25). When protectin is mixed with GPE, it spontaneously incorporates into the cell membrane via its hydrophobic lipid anchor. The full activity of protectin requires the presence of its glycolipid anchor as the soluble molecule is approximately a 200-fold less efficient inhibitor of cell lysis than the lipid-anchored protectin (26). In binding assays, the ^{125}I-labeled T47D-protectin bound to soluble SC5b-9 complexes, similarly as has been shown earlier for erythrocyte protectin (12) and soluble urinary protectin (25).

Using a specific anti-protectin mAb and F(ab')$_2$ fragments thereof, it was possible to inactivate the functional activity of protectin on cultured breast cancer cells. In complement-mediated cell lysis experiments, the maximum lysis of MCF7 cells was 30 to 60% depending on the conditions used. The fact that total lysis was not achieved suggests that a) a proportion of the cells can resist complement killing more effectively, b) the cells have protection mechanisms other than CD59 expression and/or c) that a more effective complement treatment protocol (higher doses, longer incubation times) should be used in the experiments. When studied by indirect IF microscopy using a mouse mAb against DAF (BRIC216) or MCP (GB24), both regulators were found to be expressed by MCF7 and T47D cells, although the expression of DAF was relatively weak on T47D cells. Expression of functionally active protectin on breast cancer cells shows that it has a role in protecting the malignant cells against lysis by the homologous C system and strongly suggests a similar function for it *in vivo*.

The potential uses of monoclonal antibodies in cancer therapy include targeting of various toxic molecules, complement or cytolytic cells to tumor cells. mAb have

been used in the therapy of lymphomas (27) and melanomas (28) with a limited success. In the latter study, intravenously administered monoclonal anti-GD3-ganglioside mAb was observed to become deposited on solid melanoma tumors and lead to lymphocyte and mast cell infiltration as well as to local C deposition. These results demonstrate that it is possible to launch an *in vivo* C attack against tumor cells recognized by antibodies. However, for effective killing of the tumor cells, their resistance to C must be overcome.

According to our results, the cultured breast cancer cells are protected against the C membrane attack, at least partly, by expressing protectin. In earlier studies Chorvath *et al.* (29) observed protectin on a cultured breast tumor cell line, and Kumar *et al.* (30) detected protectin mRNA in the BT20 breast tumor cell line. In the present study, protectin was strongly expressed in all the examined breast tumors including malignant cancers and benign fibroadenomas. As protectin is also expressed in the normal ductal epithelia of the mammary gland, its expression pattern is probably determined by the cellular origin of the tumor. The neutralization of protectin selectively on tumor cells, while saving the "bystander" cells, poses a problem that we have recently attempted to solve by targeting the anti-protectin mAb to the tumor cells with the help of the biotin-avidin system (31). Biotinylation of the anti-protectin mAb converts it into a nonactivator of C (32) and may facilitate its use as an adjuvant antibody in tumor-specific immunotherapy. As a nonactivator of C, the biotinylated anti-protectin mAb probably does not cause significant damage to bystander cells, although it retains its ability to neutralize protectin on the surface of tumor cells when attracted to their surfaces by an avidin-conjugated tumor-specific mAb.

In the present study a mAb against HER2/c-*erb*B-2 epidermal growth factor receptor did not sensitize MCF7 or T47D cells sufficiently to initiate a lytic C attack. On the other hand, the rabbit polyclonal antibody was an efficient inducer of complement lysis, which was enhanced by the anti-protectin mAb. For sensitization of breast cancer cells to C lysis, the choice of an appropriate antibody is thus a critical factor. A majority of the mAb produced are of murine origin and many of these are poor activators of human complement, especially the IgG1 isotype. Current possibilities to engineer mAbs should, however, allow the development of specific mAb with appropriate effector functions (33).

In conclusion, our paper demonstrates that human breast tumors strongly express the complement membrane attack inhibitor protectin (CD59). Expression of protectin apparently makes the tumor cells resistant to killing by antibodies and complement, but this resistance can be overcome by specific mAbs that neutralize the activity of protectin.

METHODS

Cell Lines and Tumor Samples

MCF7, a breast adenocarcinoma cell line (34) and T47D, a ductal carcinoma cell line (35), were obtained from American Type Culture Collection (Rockville, Maryland). Cells were grown in RPMI 1640 culture medium (Gibco, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2 mM L-glutamine and antibiotics (10 units/ml of penicillin and 100 μg/ml of streptomycin) at 37° C in humidified air with 5% CO_2. Human breast tumor samples were kindly provided by Prof. I. Virtanen (Department of Anatomy, University of Helsinki). The tumors were surgically removed, snap-frozen and sectioned (5 μm) in a cryostat. Histologic diagnoses of the tumors were determined from hematoxylin-eosin stained sections.

Antibodies and Sera

Rat hybridoma cell line producing the YTH53.1 anti-protectin mAb (IgG2b) and YTH89.1 (anti-glycophorin A) mAb were kindly provided by Prof. H. Waldmann (Department of Pathology, University of Cambridge, United Kingdom). BRIC216 (anti-DAF) and BRIC229 (anti-protectin) mouse mAb (IgG2b) were purchased from Bio-Products Laboratory (Bristol, United Kingdom). GB24 (anti-MCP) mAb was a kind gift of Dr. J. P. Atkinson (Washington University, St. Louis, Missouri). Rabbit polyclonal antisera against protectin (R2) or MCF7 cells (S2) were raised by immunizing rabbits three times with soluble urinary protectin or 10^7 heat-killed MCF7 cells intramuscularly, respectively. IgG fractions were prepared by protein G affinity chromatography (Pharmacia-LKB Biotechnology, Uppsala, Sweden). A mouse monoclonal antibody, designated clone 528 (IgG2a), against the extracellular domain of the epidermal growth factor receptor HER2/c-*erb*B-2 (36) was from Oncogene Science Inc. (Mineola, New York). NHS was obtained from healthy laboratory personnel and stored in small aliquots at −70° C.

Preparation of F(ab')$_2$ Fragments

YTH53.1 IgG2b was purified from cell culture medium using a protein G Sepharose 4 Fast Flow affinity column (Pharmacia). The immunoglobulin was eluted from the column with 0.1 M glycine/HCl, pH 2.7, and dialyzed against 0.1 M sodium phosphate, pH 7.8. The F(ab')$_2$ fragments were prepared by digesting 20 mg of the concentrated IgG with endoproteinase Glu-C from *S. aureus* strain V8 (Sigma Chemical Company, St. Louis, Missouri) at a final enzyme/protein ratio of 1/30 (w/w) (37). The F(ab')$_2$ fragments were purified by gel filtration on a Superose 12 column (Pharmacia) equilibrated with phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.2 mM Na_2HPO_4 and 0.02% NaN_3, pH 7.4). Fractions were analyzed by SDS-PAGE and those containing F(ab')$_2$ fragments (with no traces of intact heavy chain) were pooled and concentrated to 4 mg/ml.

Immunofluorescence Microscopy

Cells were harvested with Versene/EDTA (Gibco), washed twice with PBS and mixed with PBS containing 0.5% bovine serum albumin (BSA). The first antibody was added at 20 μg/ml and the suspension was incubated for 30 minutes on ice. After two washes with ice-cold BSA/PBS the fluorescein isothiocyanate-conjugated secondary antibodies (Dakopatts, Copenhagen, Denmark) diluted in BSA/PBS were added and the cells were incubated for another 30 minutes on ice. After three washes, the cells were fixed with 3% *p*-formaldehyde. Staining was controlled by omitting the first antibody and by using antibodies with no known reactivity with the target cells. Cryostat sections of the tumor tissues were stained similarly except that before antibody treatment, the sections on microscope slides were fixed for 5 minutes with cold (−20° C) acetone. After fixation, the slides were incubated with the diluted antibodies in a humidified chamber.

PURIFICATION OF TUMOR CELL PROTECTINS

YTH53.1 was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) at 2.5 mg IgG/ml gel. To purify protectin from MCF7 and T47D cells, the cells were grown in tissue culture flasks until confluent, harvested with Versene/EDTA, washed twice with PBS and solubilized during a 20-minute incubation in ice-cold PBS containing 60 mM n-octyl-β-D-glucopyranoside, 17 μg/ml of benzamidine, 1 μg/ml of antipain and 1 mM iodoacetamide (Sigma). Insoluble material was removed by centrifugation (18,000 × g, for 5 minutes) and the supernatant applied on the YTH53.1-Sepharose affinity column equilibrated with PBS. The unbound material was removed by washing with PBS. Bound protein was eluted with 0.1 M glycine/HCl, pH 2.7, containing 60 mM n-octyl-β-D-glucopyranoside. The pH of the collected fractions was set to neutral with 1.0 M Tris/HCl, pH 9.0. Fractions containing protein were pooled, dialyzed against 0.1% NP40 in PBS and concentrated using Centricon 10 microconcentrators (Amicon, Inc., Beverly, Massachusetts). The affinity-purified protectin from T47D cells was radiolabeled with [^{125}I]sodium (to a specific activity of 9.2×10^6 cpm/μg) using the Iodogen method (Pierce Chemical Company, Rockford, Illinois).

SDS-PAGE AND IMMUNOBLOTTING

Proteins were subjected to a 15% SDS-PAGE slab gel (38) and transferred to a nitrocellulose filter, pore size 0.2 μm (Bio-Rad Laboratories, Richmond, California), according to the method of Towbin *et al.* (39). After blocking nonspecific binding sites with 3% BSA, the nitrocellulose strips were incubated with YTH53.1 (19 μg/ml) or R2 (1/400) antibodies in 3% BSA/PBS for 1 hour. The primary antibodies were omitted from the control strips. After washing with PBS (3 times for 10 minutes), the bound antibodies were visualized using an appropriate biotinylated anti-IgG secondary antibody and avidin-alkaline phosphatase conjugate (Vectastain ABC-AP, Vector Laboratories Inc., Burlingame, California).

INCORPORATION OF PROTECTIN INTO CELL MEMBRANES AND INHIBITION OF COMPLEMENT LYSIS

Protectin extracted from T47D cells was incorporated into rabbit erythrocyte membranes by incubating (30 min, +37° C) radiolabeled T47D-protectin (17,000 cpm) with 2×10^7 erythrocytes in 50 μl of PBS containing 60 μM of β-D-glucopyranoside. The amount of T47D-protectin incorporated into erythrocytes was determined after washing the cells twice with PBS. For complement lysis tests, various amounts of T47D-protectin were incorporated into GPE, which were subsequently incubated with NHS (1/24 dilution) for 20 minutes at 37° C in 80 μl of PBS. PBS was added to each tube (up to 1 ml) and hemolysis was determined from OD_{412} values of the supernatants after centrifugation. For total lysis, water was added instead of PBS. Background lysis was obtained from cells treated with PBS instead of NHS.

COMPLEMENT-MEDIATED CYTOTOXICITY TEST

To label target cells (MCF7, T47D) with [^{51}]chromium, the cells were harvested and washed three times with RPMI 1640 medium. 10^8 cells were mixed with 25 μCi of sodium [^{51}]-chromate (Amersham, United Kingdom) in 150 μl of RPMI 1640 medium and incubated for 1 to 2 hours at 37° C with occasional shaking. To remove unbound [^{51}]Cr, the cells were washed twice with RPMI 1640 medium and incubated for a further 30 minutes at 37° C in 500 μl of RPMI 1640 medium. The washing cycle was repeated and the cell number adjusted to 2×10^6/ml. Duplicate aliquots (50 μl) of the [^{51}]Cr-labeled cells were incubated with appropriate antibody dilutions (70 μl) for 15 minutes at room temperature and with 50 μl of NHS for 30 minutes at 37° C. Total lysis was estimated by treating the cells with 0.1% NP40 in separate tubes. Unlysed cells were pelleted at 500 × g for 5 minutes. Eighty-five microliters of the supernatants from each tube were collected for the measurement of radioactivity released. [^{51}]Cr release in the absence of added reagents was taken as background.

BINDING OF T47D-PROTECTIN TO THE TERMINAL COMPLEMENT COMPLEXES

[^{125}I-labeled T47D-protectin (10^5 cpm) was incubated with NHS (60 μl) and 4% inulin for 60 minutes at 37° C. In controls, either NHS or inulin were omitted from the reaction mixture. To separate unbound and terminal complement complex-bound [^{125}I-protectin, the mixture was layered on top of a 10 to 50% sucrose gradient and ultracentrifuged for 17 hours at 200,000 × g using a SW50.1 rotor (Beckman Instruments, Palo Alto, California). Two hundred microliter fractions were collected and counted for radioactivity.

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We would like to dedicate this paper to our dear colleague, Dr. Elina Paalasmaa, who, at the age of 35, died of breast cancer.

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Review

Obstacles to cancer immunotherapy: expression of membrane complement regulatory proteins (mCRPs) in tumors

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Abstract

Monoclonal antibodies (mAbs) are being increasingly used in cancer therapy owing to their ability to recognize specifically cancer cells and to activate complement- and cell-mediated cytotoxicity and/or to induce growth arrest or apoptosis. The therapeutic potential of anticancer antibodies is significantly limited due to the ability of cancer cells to block killing by complement. Of the multiple resistance strategies exploited by cancer cells, the expression of membrane complement regulatory proteins (mCRPs), such as CD46 (membrane cofactor protein (MCP)), CD55 (decay-accelerating factor (DAF)), CD35 (complement receptor type-1 (CR1)) and CD59, has received most attention. CD46, CD55 and CD35 block the complement cascade at the C3 activation stage and CD59 prevents assembly of the membrane attack complex of complement (MAC). These proteins protect normal tissues from accidental injury by activated complement, but also confer resistance on cancer cells, thereby limiting the effect of complement-fixing monoclonal antibodies. Expression of mCRPs on malignant cells is highly variable, yet there is clear indication that certain tumors express higher mCRP levels than the normal tissue from which they have evolved. mCRP level of expression and cellular location may also vary during malignant transformation and between differentiated and undifferentiated tumors. Neutralizing anti-mCRP mAbs have been used in vitro to elucidate the significance of mCRP expression to the tumor complement resistance phenotype. In general, CD59 appears to be the most effective mCRP protecting tumor cells from complement-mediated lysis. Nevertheless, it acts additively, and in certain tumors even synergistically, with CD55 and CD46. It is envisaged that treatment of cancer patients with mCRP blocking antibodies targeted specifically to cancer cells in combination with anticancer complement-fixing antibodies will improve the therapeutic efficacy.

Keywords: Complement; mCRP; CD46; CD55; CD59; Cancer; Immunotherapy

1. Introduction: complement and its regulation by membrane complement regulatory proteins (mCRPs)

The complement system is a major component of the innate immune system. It efficiently protects the host from pathogenic microorganisms, contributes to immune complex regulation and represents an important link between the innate and the specific immune system (for review, see Walport, 2001a,b). Complement comprises a group of more than 30 proteins, which participate in a cascade-like activation process, serve as control proteins or act as cellular receptors. Activation of the central component, C3, may occur through three different pathways, the antibody-dependent classical pathway as well as the two phylogenetically older antibody-independent pathways, the alternative and the lectin pathways. Each of the activation pathways leads to formation of the terminal C5b-9 membrane attack complex of complement (MAC) (Muller-Eberhard, 1986). During MAC formation it is inserted into the surface membrane, leading either to cell destruction or, in sublytic doses, to cell activation (Morgan, 1989; Jurianz et al., 1999b). Complement activation elicits a number of biological effects, such as recruitment and degranulation of leukocytes, smooth muscle contraction, and increase of vascular permeability. Complement activation also induces proinflammatory conditions that affect cell surface molecules on leukocytes as well as on endothelial cells. Thus, effector functions arising from complement activation may harm the host by inducing inflammatory tissue destruction. Complement activation links to antibody-dependent cellular cytotoxicity (ADCC) through the interaction of iC3b with CR3 (CD11b/CD18) on mononuclear phagocytes and natural killer (NK) cells and activation of complement-dependent cellular cytotoxicity (CDCC) (Perlmann et al., 1981; Bara and Lint, 1987).

Abbreviations: GPI, glycosyl phosphatidylinositol; mAb, monoclonal antibody; MAC, membrane attack complex of complement; mCRP, membrane complement regulatory protein.

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A balanced action of soluble (C1 inhibitor, C4b binding protein, factors H and I, clusterin and vitronectin) as well as membrane-bound regulatory proteins (CD35, CD46, CD55 and CD59) restricts the action of complement at critical stages of the cascade reaction (Morgan and Harris, 1999). Expression of the membrane complement regulatory proteins protects normal cells and tissues but also malignant cells from complement attack. CD35 (complement receptor type-1 (CR1)), CD46 (membrane cofactor protein (MCP)) and CD55 (decay-accelerating factor (DAF)) contribute to the control of C3 activation, which is the pivotal stage in the early complement cascade, whereas CD59 interferes with the assembly of the MAC.

CD35 acts as a cofactor for factor I (fI) in cleavage of C3b first to iC3b and subsequently into C3c and C3dg (Fearon, 1979; Medof et al., 1982; Medicus et al., 1983). CD35 also binds to C4b, facilitating its degradation into C4c and C4d, even though affinity of CD35 to C4b is lower than for C3b (Iida and Nussenzweig, 1983). Beside a cofactor activity, CD35 interferes with complement activation by accelerating the decay of the C3/C5 convertases (Iida and Nussenzweig, 1983). As its name suggests, CD35/CR1 also functions as a receptor for binding immune complexes or microorganism, which are opsonized with C3b or C4b. CD35 on erythrocytes plays an important role in transport of immune complexes to the reticuloendothelial system, where degradation takes place (Schifferli et al., 1986). CD35 on leukocytes facilitates phagocytosis of opsonized microorganisms (Ehlenberger and Nussenzweig, 1977). CD35 is mainly detected on circulating cells, such as erythrocytes and most types of leukocytes (Lay and Nussenzweig, 1968; Fearon, 1980; Tedder et al., 1983; Wilson et al., 1983; Fischer et al., 1986). A soluble form of CD35, which is probably released from the surface of leukocytes, is found in the blood stream (Hamer et al., 1998). The plasma concentration of this circulating complement regulator was found to be increased in certain malignancies (Pascual et al., 1993). *CD35* gene is located in chromosome 1q32 and the protein belongs to the regulators of complement activation (RCA) protein family, whose members contain characteristic short consensus repeat (SCR) domains (Hourcade et al., 1989; Kristensen et al., 1987).

Like CD35, CD46 has cofactor activity for fI-mediated breakdown of C3b and C4b, but no decay-accelerating activity (Kojima et al., 1993). Interestingly, in the absence of fI, CD46 can act inversely and stabilize C3 convertases (Seya and Atkinson, 1989). CD46 is implicated more in the control of the alternative pathway convertase than of the classical pathway convertase (Kojima et al., 1993). CD46 is found on virtually every cell type (Johnstone et al., 1993), except for human erythrocytes (Seya et al., 1988). An abundant expression of CD46 was detected in the genital tract, where it seems to be important for fertility (Fenichel et al., 1990; Hunt and Hsi, 1990). Soluble forms of this membrane protein have been isolated from biological fluids like blood and tears (Hara et al., 1992b; Seya et al., 1995; McLaughlin et al., 1996). CD46 also belongs to the RCA protein family with gene location in the RCA cluster in chromosome 1q32 (Lublin et al., 1988). An interesting feature of MCP is the presence of different isoforms on the same cell, which are probably generated by alternative splicing (Post et al., 1991). In contrast to CD55 and CD59, both CD35 and CD46 are transmembrane proteins with a cytoplasmatic domain.

As reflected by its name, CD55/DAF triggers the decay of both C3 and C5 convertases (Nicholson-Weller and Wang, 1994). It preferentially inactivates the classical pathway convertase C4b/C2a. In contrast to CD35, the affinity of CD55 to C3b or C4b is very low, but strongly increases once the convertase is assembled (Pangburn, 1986). This enables "recycling" of DAF following the decay of the convertase complex (Morgan and Harris, 1999). CD55 is widely distributed (Nicholson-Weller et al., 1985), but was not detected on NK cells and some T lymphocytes (Nicholson-Weller et al., 1986; Tomita et al., 1991). A soluble version of CD55 is abundantly found in different biological fluids (Medof et al., 1987b), as well as in the subendothelial extracellular matrix (ECM) (Hindmarsh and Marks, 1998). As an additional member of the RCA protein family, CD55 is also comprised of SCR domains (Medof et al., 1987a) and the gene, which contains 11 exons, is also found in chromosome 1q32 (Post et al., 1990). CD55 is a glycosyl phosphatidylinositol (GPI)-anchored integral membrane protein (Medof et al., 1986; Davitz et al., 1986), which is able to insert into membranes of foreign cells (Medof et al., 1984). Alternative splicing of CD55 mRNA generates a GPI-deficient molecule with a hydrophilic carboxy terminus (Morgan and Harris, 1999).

Upon strong complement activation and initiation of MAC assembly, CD59 represents the last line of defense as it can bind to C8 and C9 and prevent C9 polymerization (Meri et al., 1990). CD59 could be detected on all circulating cells and in most tissues (Davies et al., 1989; Stefanova et al., 1989; Nose et al., 1990; Meri et al., 1991). Like CD55, CD59 is also attached to the cell surface by a GPI anchor. Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal disorder, which is characterized by the inability of some hematological stem cells to express GPI-linked proteins. Erythrocytes of affected individuals show a severe phenotype with abnormal high sensitivity to complement attack, pointing to the important role of the GPI-linked complement regulators CD55 and CD59 in the protection of host cells (Parker, 1991). A fluid phase form of CD59, which is found in seminal plasma and breast milk, retains its GPI anchor, which allows attachment to foreign cell surfaces (Rooncy et al., 1993; Hakulinen and Meri, 1995), whereas urine CD59 is devoid of the GPI anchor (Lehto et al., 1995). The sequence of the *CD59* gene shares no significant homology to the other complement regulators (Sawada et al., 1990). The three-dimensional protein structure of CD59 is strongly influenced by 10 cysteines, which form five intramolecular disulfide bonds creating several loop structures (Sugita et al., 1993). This cysteine-rich sequence motif of CD59

might be one reason for the apparent stability of the protein (Morgan and Harris, 1999). The *CD59* gene was mapped to chromosome 11p13 (Bickmore et al., 1993).

2. Interaction of cancer cells with complement: activation and regulation

From in vivo and in vitro observations described below, there is sufficient basis to propose that certain cancers activate, directly or indirectly, the autologous complement system. Various types of complement abnormalities have been described in cancer patients. Thus, in vivo classical pathway activation was described in patients with chronic lymphatic leukemia (CLL) who have low serum levels of several complement proteins and increased concentrations of circulating C1r–C1s–C1 inhibitor complexes (Fust et al., 1987; Hidvegi et al., 1989; Schlesinger et al., 1996). After a long follow-up of 43 CLL patients, Varga et al. (1995) demonstrated a positive correlation between the length of survival of CLL patients and their initial classical pathway levels and suggested that complement measurements can have a clinical value. CLL-associated hypocomplementemia probably contributes to the increased incidence of infection in these patients (Heath and Cheson, 1985). In contrast, complement levels were found normal or even elevated in patients with various hematological neoplasia (Southam and Siegel, 1966; Batlle Fonrodona et al., 1979; Minh et al., 1983). No correlation was found between the clinical course and increased plasma levels of complement components (Batlle Fonrodona et al., 1979). Higher complement hemolytic activity and C3 levels were observed in serum samples from children with neuroblastoma (Carli et al., 1979). Similarly, elevated complement levels were reported in patients with lung (Nishioka et al., 1976; Gminski et al., 1992), digestive tract (Maness and Orengo, 1977) and brain (Matsutani et al., 1984) tumors. It was hypothesized that the complement system was elevated in tumor-bearing patients to compensate for a depressed cell-mediated immune response (Matsutani et al., 1984). The opposite phenomenon was observed in patients with advanced-stage metastatic brain tumors who had reduced complement titers (Matsutani et al., 1984). Reduced CH50 and C3 (but not C4) were described in subjects with breast, gastric and colon–rectum carcinomas (Mangano et al., 1984). Following surgery, the CH50 level in these patients' sera returned to normal, and declined again in those patients developing metastases or approaching the terminal phase. Hauptmann et al. (1979) reported of a patient with extensive lymphosarcoma of the spleen having low levels of early acting complement components, and recovering to normal levels following splenectomy. In the latter report it was demonstrated that tissue pieces of the lymphosarcoma could activate and consume complement ex vivo. Yet, it is still too early to conclude that tumor cells directly activate in vivo autologous complement. It is possible that the complement system of cancer patients is activated indirectly by immune complexes, infectious agents or substances generated within a tumor mass.

Support to the claim that certain tumor cells activate complement in situ comes from few analyses of complement deposition within the tumor tissue. Deposits of C3, C4 and C5b-9 were found in breast (Niculescu et al., 1992) and papillary thyroid carcinoma (Yamakawa et al., 1994; Lucas et al., 1996). The extent of local complement activation within tumor masses or on circulating tumor cells deserves further analysis. In vitro studies of tumor cell lines have demonstrated that human tumor cells can activate to some degree human complement but are rather resistant to lysis. Thus, oat cell carcinoma (Okada and Baba, 1974) and Raji B lymphoblastoid cells (Budzko et al., 1976; Theofilopoulos and Perrin, 1976) were shown to activate the alternative human complement pathway in the absence of antibodies. C3 deposition on B lymphoblastoid cells was enhanced upon treatment with INFγ or TNFα (Ycfenof et al., 1991). Kinetics of Raji cell lysis was very slow unless the cells were pretreated with the metabolic inhibitor puromycin (Baker et al., 1977; Schreiber et al., 1980). It is well accepted today that enhancement of complement activation and lysis of tumor cells by homologous complement can be readily achieved, at least in vitro, by coating tumor cells with potent complement-fixing antibodies and by inhibiting various metabolic processes within the tumor cells. As discussed below, blocking of the protective activity of membrane complement regulatory proteins is also necessary to amplify a weak spontaneous process of complement activation into an efficient lytic complement attack on homologous tumor cells.

Neoplastic transformation may be accompanied by increased capacity of the cells to activate complement (McConnell et al., 1978). Hence, to survive in vivo, cancer cells must develop means to resist complement attack. Metabolically active nucleated cells are more resistant to killing by complement than erythrocytes (reviewed by Ohanian et al., 1978; Morgan, 1989). A sufficient and well-controlled comparison between normal and neoplastic cells is still lacking and it would still be unfounded to generalize and to claim that upon malignant transformation, neoplastic cells acquire enhanced resistance to complement. Yet, it is well established today that tumor cells resist homologous complement attack by employing a collection of protective strategies (reviewed by Jurianz et al., 1999b). Most of the resistant mechanisms are probably also utilized by normal tissues to resist accidental cell damage following local activation of complement. Perhaps the best example is the elimination of the MAC from the plasma membrane by endocytosis and by vesiculation. This phenomenon was described in neutrophils (Campbell and Morgan, 1985), oligodendrocytes (Scolding et al., 1989) and platelets (Sims and Wiedmer, 1986), but also in Ehrlich ascites tumor cells (Carney et al., 1985), U937 and K562 cells (Morgan, 1992).

Resistance of tumor cells to complement-mediated lysis depends on extracellular and intracellular factors (reviewed

in Jurianz et al., 1999b). Extracellular protectors limit the quantity of complement proteins that are deposited on the surface of target cells. They interfere with the cascade of complement activation at specific points and counteract activated complement proteins. Extracellular protection is provided by: (1) mCRP, the subject of this review; (2) soluble complement inhibitors released by tumor cells to the microenvironment, such as C1 inhibitor, factor H and factor H-like protein (Jurianz et al., 1999b; Junnikkala et al., 2000, 2002; Ziegler and Kirschfink, manuscript in preparation); (3) ecto-proteases (e.g. Ollert et al., 1990; Jurianz et al., 2001); (4) sialic acid residues (Jacobsen, 1982; Donin et al., 2003); and (5) ecto-protein kinases (Nilsson Ekdahl and Nilsson, 1997; Paas et al., 1999). Intracellular protectors reduce the extent of damage inflicted by the MAC, facilitate repair processes and eliminate the MAC from the cell surface. These mechanisms are poorly defined, yet some pieces of this complex puzzle have been identified. Factors that are known to have protective capacity from lysis are: (1) intracellular calcium ions (Morgan and Campbell, 1985); (2) cyclic AMP (Kalinec and Austen, 1974; Boyle et al., 1976); (3) protein kinase C (Kraus and Fishelson, 2000); (4) mitogen-activated protein kinase ERK (Kraus et al., 2001); (5) heat-shock protein 70 (Fishelson et al., 2001); and (6) Bcl-2 (Contreras et al., 2002; Attali and Fishelson, submitted for publication). In addition, protein synthesis (Baker et al., 1977; Ohanian and Schlager, 1981) and membrane lipid metabolism (Papadimitriou et al., 1991) were shown to be required for survival under MAC attack. These intracellular mechanisms, that will not be discussed further here, have received much less attention than the mCRP, even though their significance to complement resistance of tumor cells is probably comparable to that of the mCRP.

3. Expression of mCRP in primary tumors and by tumor cell lines

Numerous studies have been performed on the mCRPs in primary tumors and in tumor cell lines, in an attempt to clarify their significance to cancer immunoresistance. Much data was added since the last review on this subject (Gorter and Meri, 1999). To avoid an extended and laborious listing of many of those studies, their major findings are summarized in Table 1. From this table it is evident that almost all cancers studied express at least one of the mCRPs and many express CD46, CD55 and CD59. CD35 was not included in the table as it was identified only in few analyses, such as in follicular dendritic cell tumors (Perez-Ordonez and Rosai, 1998), malignant endometrial tissue (Murray et al., 2000) and in leukemic blasts (Guc et al., 2000). The fact that most cancers, independent of their tissue origin, express at least two if not three mCRPs, is perhaps not surprising considering the wide tissue distribution of CD46, CD55 and CD59 (reviewed in Morgan and Harris, 1999). However, surprisingly, large variations in mCRP expression have been observed among various samples of same tumors within studies and between studies of the same tumor. For example, marked variation in mCRP expressions was seen among cases of breast or renal cell carcinomas (Niehans et al., 1996). This could not be correlated with complement activation and MAC deposition. Weichenthal et al. (1999) analyzed samples of 16 metastatic melanoma lesions and observed that 9 expressed both CD46 and CD59, 2 had CD59 only, 1 had CD46 only and 4 had neither CD46 nor CD59. Brasoveanu et al. (1996) studied nine melanoma cell lines and found a large heterogeneity in CD59 expression among them. The more CD59 these melanoma cells expressed, the more resistant they were to killing by anti-ganglioside monoclonal antibody (mAb) and homologous complement. In contrast, in ovarian cancer, CD55 was more heterogeneously expressed and resistance to complement correlated in these cells with level of CD55 expression (Bjorge et al., 1997a,b). In the two latter studies, treatment with anti-CD59 mAb sensitized the melanoma and ovarian carcinoma cells, respectively, to complement-mediated lysis. This will be discussed further in Section 4. It is important to note that the number of reports demonstrating increased expression of mCRP in tumors relative to the corresponding normal tissue is consistently increasing (Bjorge et al., 1994; Hofman et al., 1994; Niehans et al., 1996; Simpson et al., 1997; Varsano et al., 1995, 1998a; Blok et al., 2000; Murray et al., 2000; Nakagawa et al., 2001; Nowicki et al., 2001). For example, the expression of CD46 in primary cervix tissue was found to increase from normal to premalignant to malignant cells (Simpson et al., 1997). Li et al. (2001) examined colorectal and gastric carcinomas and osteosarcoma and found increased expression of CD55, whereas Kiso et al. (2002) found increased expression of both CD55 and CD59 in intestinal type gastric carcinoma. Overexpression of CD59 was also identified by expression profiling for pancreatic cancer (Crnogorac-Jurcevic et al., 2002).

What is causing the variability in expression of mCRP on tumor cells? One simple explanation could be: the absence of any selective force, i.e. mCRPs are constitutively produced (or not) and without any external interference, e.g. as long as the cells are not killed by complement, the level of mCRP will remain variable. Killing or opsonization by complement will select for the high mCRP expressors. Alternatively, the level of expression of each mCRP may reflect a stage in differentiation of specific tumor cells. Thus, poorly differentiated colorectal carcinoma cells expressed low or no CD59, whereas differentiated carcinoma cells expressed higher CD59 levels (Koretz et al., 1993). Similarly, differentiated gastric carcinoma strongly expressed CD59 on the luminal membrane, whereas in undifferentiated tumors, CD59 was almost absent (Inouc et al., 2002). In the latter study, both differentiated and undifferentiated gastric carcinomas had higher CD55 expression than the normal epithelium. Thirdly, the level of expression of mCRPs may be influenced by host factors, such as hormones and cytokines or growth factors released by neighboring tumor or stromal cells, in a mechanism still unclear to us. Thus, TNFα and

Table 1
Expression of CD46, CD55 and CD59 in primary tumors and tumor cell lines and secreted into the microenvironment (tumor stroma, culture supernate or extracellular matrix)[a]

Cancer	CD46			CD55			CD59			References
	Tumor	Secreted	Cell line	Tumor	Secreted	Cell line	Tumor	Secreted	Cell line	
Blastoma										
Glioblastoma			+			+			++	Junnikkala et al. (2000)
Neuroblastoma			+/±			+/±			+/±	Gasque et al. (1996), Chen et al. (2000b)
						−				Cheung et al. (1988), Gasque et al. (1996)
Malignant glioma	±		+	±		±	++		++	Maenpaa et al. (1996)
Carcinoma										
Thyroid	++			+			++			Yamakawa et al. (1994)
Prostate			+			+	++/±		++/+	Jarvis et al. (1997), Donin et al. (2003)
Breast	↑+/+		++/+/±	+	+	+/±	++/±	+	++/+	Cheung et al. (1988), Hakulinen and Meri (1994), Hofman et al. (1994), Niehans et al. (1996), Thorsteinsson et al. (1998), Jurianz et al. (1999a), Yu et al. (1999), Donin et al. (2003)
		−		−		−				Hofman et al. (1994), Niehans et al. (1996), Thorsteinsson et al. (1998)
Ovarian	++	+	↑+/++/+	±	±	+/±	++	++	++/+	Bjorge et al. (1997a,b), Peng et al. (2002), Donin et al. (2003)
Endometrial tissue	↑+			↑+/++/+			↑+			Murray et al. (2000), Nowicki et al. (2001)
Lung	↑+/+		↑+/+	↑+	+	↑+/+	↑+/+	+	↑+/+	Sakuma et al. (1993), Varsano et al. (1995, 1998a,b), Niehans et al. (1996)
		−		−		−				Niehans et al. (1996), Cheung et al. (1988)
Colorectal	++/+	±	++/+/±	↑+/±	++/+	↑+/++/+/±	↑+/+/±	+	++/±	Andrew et al. (1990), Koretz et al. (1992, 1993), Bjorge et al. (1994, 1996), Inoue et al. (1994), Mizuno et al. (1995), Niehans et al. (1996), Juhl et al. (1997), Thorsteinsson et al. (1998), Schmitt et al. (1999), Hosch et al. (2001), Li et al. (2001), Nakagawa et al. (2001), Andoh et al. (2002), Gelderman et al. (2002b)
	↓−	−		−			↓−/−			Inoue et al. (1994), Niehans et al. (1996), Thorsteinsson et al. (1998), Schmitt et al. (1999)
Pancreatic	++	±	++/+	±	+	++/±	↑+/+	+	++/±	Juhl et al. (1997), Schmitt et al. (1999), Crnogorac-Jurcevic et al. (2002)
Gastric	++/+	±	++/+	↑+/+	++/+/±	↑+/±	↑+/++	+	++/±	Juhl et al. (1997), Mikami et al. (1998), Schmitt et al. (1999), Hensel et al. (2001), Inoue et al. (2002), Li et al. (2001), Kiso et al. (2002)
				−	−		−		−	Mizuno et al. (1995), Juhl et al. (1997), Schmitt et al. (1999), Kiso et al. (2002)
Kidney	±		+	↑+/+	+	+	↑+/±	±	+	Terachi et al. (1991), Gorter et al. (1996), Niehans et al. (1996), Blok et al. (2000)
	↓−	−		−						Niehans et al. (1996), Blok et al. (2000)
Oral squamous			+			+			++	Ravindranath et al. (2000)
Skin	+			±			+			Sayama et al. (1992)
Cervical	↑+		↑+	±	↑+/+		±	+	↑+	Simpson et al. (1997), Gelderman et al. (2002a)
Hepatoma	↑+		+			+			++	Kinugasa et al. (1999), Spiller et al. (2000)

Table 1 (*Continued*)

Cancer	CD46			CD55			CD59			References
	Tumor	Secreted	Cell line	Tumor	Secreted	Cell line	Tumor	Secreted	Cell line	
Melanoma	+/±		+	+/±		+/±	+	+	++/+/±	Brasoveanu et al. (1995, 1996, 1997), Goslings et al. (1996), Blom et al. (1997), Weichenthal et al. (1999)
				−		−			−	Brasoveanu et al. (1995), Cheung et al. (1988), Weichenthal et al. (1999)
Hematological Leukemia	↑+/+		↑+/+	↑+/++/+		↑+/++/+	↑+/+/±	+	↑+/+/±	Seya et al. (1990), Fukuda et al. (1991), Hara et al. (1992a, 1995), Paloczi et al. (1995), Guc et al. (2000), Golay et al. (2001), Jurianz et al. (2001)
				AML ↓−, ALL ↓−						Guc et al. (2000)
Lymphoma	+		+	+		+/±	+		+/±	Fukuda et al. (1991), Hara et al. (1992a), Kuraya et al. (1992, 1993), Seya et al. (1994), Golay et al. (2000, 2001), Treon et al. (2001)
				NHL −/±					−	Kuraya et al. (1992), Seya et al. (1994)
Osteosarcoma			++			++			++	Li et al. (2001)

[a] Level of mCRP expression: −, no; ±, low; +, moderate; ++, high; ↓−, expression in tumor lower than in normal tissue; ↑+, expression in tumor higher than in normal tissue.

IL-1β enhanced expression of CD55 and CD59 in colon adenocarcinoma cells (Bjorge et al., 1995). TNFα, IL-1α and INFγ enhanced CD55 expression on lung cancer cell lines (Varsano et al., 1998b). TNFα, IL-1β and IL-6 have been shown to increase in vitro expression of CD55 and CD59 but decreased CD46 expression in hepatoma cells (Spiller et al., 2000). As space is limited, only few examples have been given, but in general most cytokines tested so far either enhanced or had no effect on mCRP expression, at least in vitro. Anoxic conditions may also affect tumor–complement interaction in situ by reducing CD59 level of expression (Vakeva and Meri, 1998). In addition, a variety of gene or transcription abnormalities may lead to modified expression of mCRPs (Hatanaka et al., 1996). In summary, the factors regulating in vivo the level of expression and cellular location (Niehans et al., 1996; Bjorge et al., 1997a,b) of mCRPs in cancer cells remain to be elucidated.

Soluble forms of the mCRPs have been identified in most body fluids, even under normal conditions. Soluble CD55 (sCD55) was found in urine (Medof et al., 1987b; Nakano et al., 1991), plasma, synovial fluid, saliva and cerebrospinal fluid (Medof et al., 1987b). sCD59 was identified in urine (Davies et al., 1989) and amniotic fluid (Rooney and Morgan, 1992). Sera of cancer patients contain active, soluble forms of CD46 (Seya et al., 1995). Elevated CD55 concentration in stool specimens has been proposed to have a diagnostic value for patients with colorectal cancer (Mizuno et al., 1995; Iwagaki et al., 2002). Brasoveanu et al. (1997) reported of constitutive release of sCD59 from human melanoma cells. The latter sCD59 retained its activity as well as its GPI anchor. Analysis of mCRPs in primary tumor sections disclosed also the occurrence of CD55 and/or CD59 in the stroma of breast, colorectal, lung, renal and cervical carcinomas (Niehans et al., 1996; Li et al., 2001; Gelderman et al., 2002a,b). In vitro studies demonstrated that endothelial cells, HeLa cells (Hindmarsh and Marks, 1998), osteosarcoma and colorectal cells (Nasu et al., 1998; Li et al., 2001) released CD55 in a soluble form or deposited it into their extracellular matrix. sCD59 accumulated also in culture supernates of K562 erythroleukemia cells (Jurianz et al., 2001) and of breast, ovarian and prostate carcinoma cell lines (Donin et al., 2003). Many leukemia patients (AML, ALL and CLL) show elevated plasma levels of sCR1 (Sadallah et al., 1999). Soluble complement regulators, secreted into the microenvironment have been found to bind to the tumor cell, thereby adding to the regulatory capacity of mCRP. (Junnikkala et al., 2002; Ziegler and Kirschfink, manuscript in preparation). The underlying shedding or secretory mechanism still awaits clarification, yet it appears that the soluble or ECM-attached CRPs retain their complement inhibitory activity. Therefore, they may further protect the neighboring tissue, healthy or malignant, from complement activation.

Another prove to the protective potency of CD59 from complement-mediated killing comes from overexpression experiments. Chen et al. (2000a) transfected rat CD59 into human neuroblastoma cells that were sensitive to killing by rat complement. The rat CD59-positive cells became resistant to killing by rat complement in vitro and grew faster in immune-deficient rats as compared with control cells. Similarly, overexpression of CD59 in melanoma cells by infection with a retroviral vector carrying CD59 cDNA conferred on them resistance to killing by anti-GD3 mAb and complement (Coral et al., 2000).

4. Means to overcome protection by mCRP

The use of anticancer antibodies for treatment of cancer patients has received recently much attention and interest, especially following the clinical and commercial success of mAb, such as rituximab (Rituxan) and trastuzumab (Herceptin) (Carter, 2001; Ross et al., 2003). It is likely that the therapeutic potential of monoclonal antibodies is largely impaired by the mCRPs (Juhl et al., 1997; Treon et al., 2001; Golay et al., 2001). To succeed in tumor eradication with antibody and complement, novel methods and reagents that overcome the protective capacity of mCRPs are needed. Theoretically, this may be achieved either by: (1) blockade of the regulator activity; (2) down-regulation of mCRP expression; or (3) removal of the protein from the cell surface. Specific inhibition of mCRP activity has been achieved with monoclonal antibodies directed to CD46, CD55 or CD59 that block their function. As these mAbs are usually poor activators of complement on their own, they may be used together with complement-fixing antibodies or other complement activators for testing the involvement of specific mCRPs in cell resistance. In most cases (data summarized in Table 2), anti-mCRP blocking antibodies successfully enhanced the susceptibility of tumor cells to complement-mediated lysis. For example, neutralization of CD55 in Burkitt lymphoma cells (Kuraya et al., 1992), leukemia cells (Zhong et al., 1995; Jurianz et al., 2001; Golay et al., 2001), melanoma cells (Cheung et al., 1988) and breast cancer cells (Jurianz et al., 1999a,b; Donin et al., 2003) increased their sensitivity to complement. On the other hand, anti-CD55 mAb had no effect on killing of renal carcinoma cells (Gorter et al., 1996) and a weak effect on prostate and ovarian carcinoma cells (Donin et al., 2003). Interestingly, despite the fact that the latter carcinoma cell lines expressed significant levels of CD55, blocking of this mCRP was not sufficient to sensitize them to complement-mediated lysis. Similarly, blocking CD46 with neutralizing mAb did not significantly affect complement sensitivity of erythroleukemic cell line K562 and of cervix carcinoma cell lines (Jurianz et al., 2001; Gelderman et al., 2002a). However, neutralization of CD59 with mAb produced efficient sensitization to complement-mediated lysis of neuroblastoma cells (Gasque et al., 1996), leukemic cells (Jurianz et al., 2001; Golay et al., 2001), breast (Jurianz et al., 1999a,b; Donin et al., 2003), ovarian (Bjorge et al., 1997a,b; Donin et al., 2003), renal (Gorter et al., 1996) and prostate carcinoma cells

Table 2
Effect of neutralizing anti-mCRP mAbs on in vitro killing of tumor cells by complement[a]

Cancer	αCD46 mAb	αCD55 mAb	αCD59 mAb	Combined mAb	References
Blastoma					
Glioblastoma			−	−	Junnikkala et al. (2000)
Neuroblastoma	− (b)	+ (b)	+ (a, b)		(a) Gasque et al. (1996), (b) Chen et al. (2000b)
Glioma			+/−		Macnpaa et al. (1996)
Carcinoma					
Prostate	− (b)	± (b)	+ (a, b)	++ (b)	(a) Jarvis et al. (1997), (b) Donin et al. (2003)
Breast	− (b, c)	+ (b, c)	+ (a–c)	++ (b, c)	(a) Hakulinen and Meri (1994), (b) Jurianz et al. (1999a), (c) Donin et al. (2003)
Ovarian	± (b)	± (b)	+ (a, b)	++ (b)	(a) Bjorge et al. (1997a), (b) Donin et al. (2003)
Lung	−	−	±		Varsano et al. (1998a)
Colorectal		+ (b)	+ (a)		(a) Bjorge et al. (1994), (b) Bjorge et al. (1996)
Kidney	− (a)	− (a), + (b)	+ (a)		(a) Gorter et al. (1996), (b) Blok et al. (1998)
Cervical	−	+	+	++	Gelderman et al. (2002a)
Melanoma		+ (c)	+ (a, b, d)		(a) Junnikkala et al. (1994), (b) Brasovcanu et al. (1995, 1996, 1997), (c) Cheung et al. (1988), (d) Coral et al. (2000)
Hematological neoplasia	− (a, e), ± (c, f, h)	+ (a, b, d, e, g, h), ± (c, f)	+ (e–i), ± (c)	++ (a–c, g)	(a) Matsumoto et al. (1991), (b) Kuraya et al. (1992), (c) Harris and Morgan (1995), (d) Zhong et al. (1995), (e) Golay et al. (2000), (f) Harjunpaa et al. (2000), (g) Golay et al. (2001), (h) Jurianz et al. (2001), (i) Treon et al. (2001)

[a] Magnitude of mAb effect on lysis: −, no effect; ±, small effect; +, enhanced killing; ++, large enhancement.

(Jarvis et al., 1997; Donin et al., 2003). CD55 and CD46 were reported to be synergistic in complement regulation by CHO cells (Brodbeck et al., 2000). This has been confirmed and extended to breast, ovarian and prostate carcinoma cell lines by using a mixture of optimal and suboptimal concentrations of neutralizing mAbs directed to CD46, CD55 and CD59 (Donin et al., 2003), suggesting some type of cooperativity among CD46, CD55 and CD59.

Besides using blocking anti-mCRP mAb, attempts were made to modulate the level of expression of mCRP by treatments with cytokines or growth factors. As mentioned also in Section 3, most cytokines tested so far either enhanced or had no effect on mCRP expression. For cancer therapy, the aim should be to identify a treatment that reduces mCRP expression. Only few such down-regulators have been described. Thus, expression of CD59 and CD46 was decreased in hepatoma cells upon treatment with INFγ (Spiller et al., 2000). Recently, Andoh et al. (2002) suggested that butyrate, generated by bacterial degradation of dietary fiber in the colon, increases in vitro sensitivity of colon cancer cells to complement-mediated injury by down-regulating CD55 expression. Similarly, fludarabine treatment was shown to down-regulate the level of CD55 expression in B lymphoma cells and to increase their lysis by the anti-CD20 rituximab antibody and human complement (Di Gaetano et al., 2001). Less useful perhaps for clinical use is the removal of CD55 and CD59, two GPI-linked mCRPs, with GPI-phospholipase C (GPI-PLC). Lysis of melanoma cells (Brasovcanu et al., 1996; Goslings et al., 1996), lung cancer cells (Varsano et al., 1998a) and cervical carcinoma (Gelderman et al., 2002a) increased following treatment with GPI-PLC. Nevertheless, it is important to be aware that GPI-PLC will remove also other surface proteins besides CD55 and CD59 and may cause some non-specific plasma membrane perturbations, therefore the enhanced sensitivity is not necessarily related to reduced expression of CD55 and CD59.

Part of the antitumor effect of rituximab, an anti-CD20 chimeric monoclonal antibody used in the therapy of non-Hodgkin's B-cell lymphoma, has been ascribed to its capacity to bind C1q, activate complement and eventually kill the cells (Idusogie et al., 2000, 2001). The heterogeneity of the response to rituximab in different patients might have been related to mCRP expression on their tumor cells. However, a relationship between the level of expression of mCRP in follicular lymphoma or CLL cells or these cell sensitivity to in vitro complement-mediated lysis and the clinical outcome of rituximab treatment was not found, therefore their predictive value was questioned (Weng and Levy, 2001; Bannerji et al., 2003). Nevertheless, the additional application of blocking anti-CD55 or anti-CD59 antibodies increased rituximab-induced complement-mediated killing in various lymphoma cell lines as well as in fresh patients-derived follicular non-Hodgkin's lymphoma and B-cell chronic leukemia samples (Golay et al., 2000, 2001; Harjunpaa et al., 2000; Treon et al., 2001). The latter findings were supported by those of Bannerji et al. (2003) demonstrating increased CD59 expression on CLL cells that were not cleared from the blood at completion of rituximab treatment. In an attempt to improve complement targeting after rituximab treatment, an antibody directed to iC3b was added to rituximab-sensitized complement treated tumor cells (Kennedy et al., 2003). Accumulation

of C3b molecules onto the tumor cells and their killing by complement was largely enhanced. This novel treatment strategy may also promote targeted activation of CDCC and ADCC on tumor cells. In this context, studies of Ross et al. (1999) are of potential clinical importance. To override natural mechanisms of self-protection of iC3b-opsonized tumor cells, NK cells were primed with β-glucan. This treatment not only led to an improvement of CDCC but also to a tumor-localized secretion of proinflammatory cytokines. Targeted activation of complement-mediated lysis and CDCC may be also achieved with heteroconjugates composed of antitumor antibodies and cobra venom factor (CVF) (Vogel and Muller-Eberhard, 1981), C3b (Reiter and Fishelson, 1989) or iC3b (Yefenof et al., 1990). Upon conjugation of CVF or C3b to 17-1A, a mAb directed against the 17-1A antigen/epithelial cell adhesion molecule (Ep-CAM), Gelderman et al. (2002b) observed an up to 13-fold increase in C3 deposition correlated with augmented complement-mediated lysis of colorectal carcinoma cells, as compared to the 17-1A mAb alone.

As mCRP are present on the surface of normal tissues, it is important to design reagents that reach selectively mCRP on tumor cells. One such approach is the construction of bispecific mAb consisting of one F(ab) moiety directed to a tumor specific antigen and another one directed to an mCRP. However, to avoid significant binding to normal tissue, the tumor directed F(ab) moiety needs to be of high affinity (Gelderman et al., 2002a). Bispecific antibodies which recognize tumor antigens and mCRP, such as CD55 (Blok et al., 1998) or CD59 (Harris et al., 1997) induced effective tumor cell killing with only marginal effects on bystander cells. Gelderman et al. (2002b) generated a bispecific mAb directed against both HLA class I and CD55. Anti-HLA–anti-CD55 bispecific mAb, in presence of normal human serum, increased C3c deposition on colorectal cells as compared with anti-HLA mAb alone and with a mixture of anti-HLA and anti-CD55 mAbs. Anti-Ep-CAM–anti-CD55 bispecific antibody also increased C3 deposition on cervical and colorectal carcinoma cells, as good and even better than anti-Ep-CAM mAb conjugated with CVF or C3b (Gelderman et al., 2002a,b).

Another interesting option in immunotherapy is the use of anti-idiotypic antibodies mimicking mCRP. The human anti-idiotypic antibody 105AD7, originally isolated from a colorectal cancer patient, not only recognizes the binding site of 791T/36 (an antibody directed to an osteosarcoma cell line) but also mimics CD55 (referred to as gp72) (Austin et al., 1989). Immunization of both mice and rats with 105AD7 resulted in the generation of antibodies that bind to CD55 (Austin et al., 1991). In recent studies where patients received 105AD7 at diagnosis and prior to tumor resection, increased infiltration of CD4, CD8 and CD56 cells and increased tumor cell apoptosis were observed relative to unimmunized control patients (Spendlove et al., 2000; Amin et al., 2000).

5. Perspectives

mCRPs impose an obvious obstacle to anticancer antibody-based therapy. This obstacle may be overcome by neutralization of mCRP activity as adjuvant therapy. To ensure that the mCRP blockers will not bind to normal tissues and increase their sensitivity to autologous complement, they ought to be conjugated to a tumor-targeting moiety, such as tumor specific antibodies. The finding that even after blocking of the mCRP, some tumors still remained resistant to complement-mediated lysis (Junnikkala et al., 2000) and that mCRP expression in vivo in cancers is largely heterogeneous with specimens lacking or having low mCRP expression (e.g. Weichenthal et al., 1999), slightly lowers the expectations from mCRP targeted therapy. On the other hand, that in many cancer patients, at least in the early stages of the disease, complement activity is elevated, raises the prospect of complement-based therapy. Another reason for optimism comes from the observation that chemotherapeutic agents enhance in vitro killing of cancer cells by antibody and complement (Segerling et al., 1975; Niculescu et al., 1992), indicating that complement-based therapy could be combined with chemotherapy. It is important to emphasize again, as described in Section 2, that cancer cells are equipped with additional resistance molecules and strategies besides mCRP. To exploit the full cytolytic potential of antibody-dependent complement-mediated cancer immunotherapy, blockade of those other protectors may be necessary. Such a combined therapeutic strategy has been recently used in vitro for killing of human leukemia and carcinoma cell lines by complement (Jurianz et al., 2001; Donin et al., 2003). Another future approach may be the targeted neutralization of mCRP by antisense or other RNA silencing strategies. Preliminary data indicates that mCRP expression in tumor cells can be significantly inhibited by antisense oligonucleotides, and this is associated with effective reduction of complement resistance (Zell and Kirschfink, manuscript in preparation). So far, most if not all mCRP neutralization experiments have been performed in vitro. It still remains to be investigated in animal models whether or not the mCRP neutralizing antibodies can block mCRP activity within tumors and promote tumor elimination.

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CD59 Expressed on a Tumor Cell Surface Modulates Decay-accelerating Factor Expression and Enhances Tumor Growth in a Rat Model of Human Neuroblastoma[1]

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ABSTRACT

It has been hypothesized that complement inhibitors expressed on the surface of tumor cells prevent effective immune-mediated clearance. Whereas there are *in vitro* data to support this hypothesis, the species-selective activity of complement inhibitors has been a hindrance to investigating the role of membrane-bound complement inhibitors in rodent models of human cancer. The CD59-positive LAN-1 human neuroblastoma cell line was significantly more sensitive to lysis by rat complement than by human complement, illustrating the species selectivity of endogenously expressed complement inhibitors. Transfection of LAN-1 cells with rat CD59, an inhibitor of the terminal cytolytic membrane attack complex, effectively protected the cells from lysis by rat complement *in vitro*. When LAN-1 cells stably expressing rat CD59 were inoculated into immune-deficient rats, the onset of tumor growth and the rate of tumor growth were significantly enhanced compared with those of control-transfected LAN-1 cells. These data show directly that the expression of a complement inhibitor on a tumor cell promotes tumor growth. Flow cytometric analysis revealed that the endogenous expression of decay-accelerating factor (DAF), an inhibitor of complement activation, was up-regulated on the surface of cells after *in vivo* growth. Of further interest, higher levels of DAF were present on CD59-transfected cells than on control-transfected cells derived from tumors. Increased DAF expression correlated with decreased complement deposition on the tumor cell surface. These results show that expression of complement inhibitors on a tumor cell has functional consequences with regard to complement deposition *in vivo* and indicate that CD59 can indirectly effect complement activation and C3 deposition *in vivo* via a link between CD59 and DAF expression.

INTRODUCTION

Normal cells are protected from inappropriate complement attack by membrane-bound complement-inhibitory proteins that either prevent complement activation or block the formation of the terminal cytolytic MAC.[3] Tumor cells also express complement-inhibitory proteins, sometimes at elevated levels, and provide tumor cells with protection from complement-mediated injury. Blocking the function of complement inhibitors expressed on the surface of tumor cells may allow effective immune-mediated clearance of some tumors and improve prospects for immunotherapy using complement-activating antitumor antibodies. Complement effector mechanisms that may be involved in host response to tumor cells include the activation and amplification of an inflammatory response, recruitment of immune effector cells, promotion and enhancement of cell-mediated lysis, and direct complement-mediated cytolysis. The major inhibitors of complement activation on human cells are DAF and MCP. These proteins regulate complement enzymatic complexes that are involved in the amplification of the cascade and the resulting generation of C3/C4 opsonizing fragments and physiologically active C3a and C5a peptides. Formation of the cytolytic and proinflammatory MAC on host cell membranes is inhibited by CD59, a glycosylphosphatidyl inositol-linked glycoprotein that binds to C8 and C9 in the assembling complex.

Complement inhibitors have been found on nearly all primary tumors and cancer cell lines that have been examined, and some studies indicate that complement-inhibitory proteins are up-regulated on tumor cells. DAF and the serum complement inhibitor factor H or related proteins have been identified as tumor-associated antigens (1, 2), and the overexpression of DAF confers a poor prognosis in colorectal cancer patients (2). *In vitro* studies have shown that complement inhibitors expressed on tumor cells can inhibit both complement opsonization and direct cytolysis by the MAC (for recent reviews of immune evasion and complement resistance of tumor cells, see Refs. 3 and 4). However, there is little information regarding how tumor-expressed complement inhibitors relate to complement deposition *in situ*, and the *in vivo* relevance of complement effector mechanisms and the importance of tumor-expressed complement inhibitors in controlling tumor growth remain largely unexplored. One reason for this is that complement inhibitor proteins (particularly CD59) are species selective, and human complement inhibitors are less effective against rat and mouse complement (5, 6). Thus, endogenous complement inhibitors expressed on the surface of human tumor cells will not provide the cells with effective protection from complement in rodent models of human cancer. Indeed, the species-selective activity of membrane complement-inhibitory proteins may be a basis for observations that complement-activating mAbs effective at causing regression of human tumors in rodents have, in most cases, proven ineffective in clinical trials.

When investigating the role of complement-inhibitory proteins in immune evasion of tumor cells *in vivo*, it is therefore relevant to study rodent complement inhibitors in rodent models of cancer. The ubiquitous and high level of expression of membrane complement inhibitors on normal tissues has not allowed for the targeted blocking of complement inhibitors (using current technologies) on tumor cells in syngeneic rodent models of cancer. In the studies described here, we investigated the effect of heterologously expressed rat CD59 on the growth of a human neuroblastoma cell line in nude rats. The neuroblastoma cell line endogenously expressed CD59, but we have previously determined on a quantitative basis that human CD59 is several-fold less effective at inhibiting rat complement compared to human complement (6). The data show for the first time *in vivo* that the complement inhibitor CD59 expressed on a tumor cell surface significantly promotes tumor growth. We also show that growth *in vivo* resulted in the up-regulation of DAF on the tumor cell surface and that the level of DAF expression was further up-regulated by the expression of functional CD59.

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[3] The abbreviations used are: MAC, membrane attack complex; DAF, decay-accelerating factor; MCP, membrane cofactor protein; mAb, monoclonal antibody.

MATERIALS AND METHODS

Cells and DNA. The LAN-1 neuroblastoma cell line was obtained from Dr. Robert Seeger (University of California at Los Angeles, Los Angeles, CA) and maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Incubation was at 37°C in 5% CO_2. cDNA encoding rat CD59 and cDNA encoding murine Ly6E were the gifts of Drs. B. P. Morgan (University of Wales, Cardiff, United Kingdom) and U. Haemmerling (Memorial Sloan-Kettering Cancer Center, New York, NY), respectively. Stably transfected LAN-1 cell populations were selected by fluorescence-activated cell sorting after the cultivation of cells in the presence of G418.

Antibodies and Complement. mAbs to human (YTH53.1) and rat (6D1) CD59 and rabbit antirat C9 polyclonal IgG were the gifts of Dr. B. P. Morgan. Human MCP mAb M75 (7) was a gift of Dr. D. M. Lublin (Washington University, St. Louis, MO). Antihuman DAF mAb 1A10 was described previously (8), and anti-GD2 3F8 mAb (9) was described previously. Goat antihuman C3 IgG cross-reactive with rat C3 was obtained from ICN Pharmaceuticals (Aurora, OH). Anti Ly6A/E mAb D7 was purchased from BD Pharmingen (San Diego, CA). FITC-conjugated antibodies used for flow cytometry were purchased from Sigma (St. Louis, MO). Normal human serum was obtained from the blood of healthy volunteers in the laboratory, and rat serum was obtained from the blood of normal and immune-deficient rats. Serum was stored in aliquots at −70°C until use.

Preparation of LAN-1 Transfectants. Rat CD59 cDNA and Ly6E cDNA were subcloned into the multiple cloning site of mammalian expression vector pCDNA3 (Invitrogen, Carlsbad, CA). DNA was transfected into 50–75% confluent LAN-1 cells using LipofectAMINE according to the manufacturer's instructions (Life Technologies, Inc., Grand Island, NY). Stable populations of LAN-1 cells expressing either rat CD59 or Ly6E were isolated by several rounds of cell sorting using either antirat CD59 mAb 6D1 or anti-Ly6A/E mAb D7 as described previously (10).

Complement Lysis Assays. Complement-mediated cell lysis was determined by both ^{51}Cr release (11) and enumeration after trypan blue staining (12), as described previously. Both methods gave similar results. Lysis assays of LAN-1 cells were performed using detached cells in both the absence and presence of antitumor complement-activating antibody. In assays in which cells were antibody-sensitized to complement, the anti-GD2 monoclonal antibody 3F8 was added at 15 μg/ml, and cells were incubated for 30 min at 4°C before the addition of rat serum. Experimental details have been described previously (13).

Flow Cytometric Analysis. Analysis of cell surface protein expression and complement protein deposition was performed by flow cytometry using appropriate antibodies (see above), as described previously (10). Primary antibodies and isotype-matched irrelevant control antibodies were used at a concentration of 10 μg/ml. Analysis was performed on cells removed from tissue culture using versene (Life Technologies, Inc.) for cell detachment and on cells isolated from excised tumors. Cell suspensions were obtained from tumors by gentle teasing of tumor tissue (in RPMI 1640/10% FCS) with scalpels, followed by low-speed centrifugation through Ficoll to remove tumor pieces and aggregates (14). Tumor-derived cells were then washed in RPMI 1640/10% FCS by centrifugation before use.

***In Vivo* Experiments.** Four-week-old male athymic *nu/nu* (nude) rats were obtained from the National Cancer Institute (Frederick, MD). The rats were housed in a clean room, and food and water were sterilized. Rats were injected s.c. in the right flank with the indicated numbers of LAN-1 cells suspended in 0.2 ml of PBS. Groups of rats received either LAN-1 cells transfected with rat CD59 or control-transfected LAN-1 cells. Control cells were transfected with Ly6E (a structural but not functional homologue of CD59) or with empty plasmid. There was no difference in tumor growth between the different control LAN-1 cells. Tumor volumes were calculated using the formula $4/3\pi r^3$ (volume of sphere). Statistical analyses were performed using the SAS system (SAS Institute Inc., Cary, NC).

RESULTS

Expression of Rat CD59 on LAN-1 Cells Confers Resistance to Rat Complement. We have previously shown that LAN-1 expresses CD59, DAF, and MCP and that the sensitivity of LAN-1 and LAN-1-derived clones to lysis by human complement can be significantly enhanced by blocking CD59 function. Blocking DAF function on LAN-1-derived clones only slightly enhanced sensitivity to human complement, whereas blocking MCP function had no effect (13). However, human CD59 is not an effective inhibitor of rat complement (6), and Fig. 1 shows that LAN-1 cells are significantly more sensitive to lysis by rat complement than lysis by human complement after sensitization by anti-GD2 3F8 mAb. LAN-1 cells express high levels of GD2 antigen, and the complement-activating properties of 3F8 mAb have been described previously (13, 15). Of note, LAN-1 cells are also lysed by rat complement in the absence of 3F8 mAb, albeit less effectively (Fig. 1*b*). These results confirm that endogenous expression of human complement inhibitors on LAN-1 cells does not provide effective protection from lysis by rat complement. Similar data were obtained with serum isolated from either normal or immune-deficient rats. Lysis of LAN-1 cells in the absence of sensitizing antibody may be due to the presence of natural endogenous complement-activating antibodies that bind to LAN-1 cells, and flow cytometric analysis of cells after incubation in heat-inactivated rat serum revealed that small amounts of rat immunoglobulin were deposited on the cell surface, supporting this possibility (data not shown).

LAN-1 cells were transfected with rat CD59, and LAN-1 cells stably expressing CD59 were isolated by cell sorting (Fig. 2). As a control for *in vivo* studies (see below), LAN-1 cells were also transfected with murine Ly6E antigen, a structural but not functional analogue of CD59, and sorted as described for rat CD59 transfectants. Fig. 3 shows that the expression of rat CD59 on LAN-1 cells significantly enhanced their resistance to lysis by rat complement, both in the absence and presence of complement-activating 3F8 mAb.

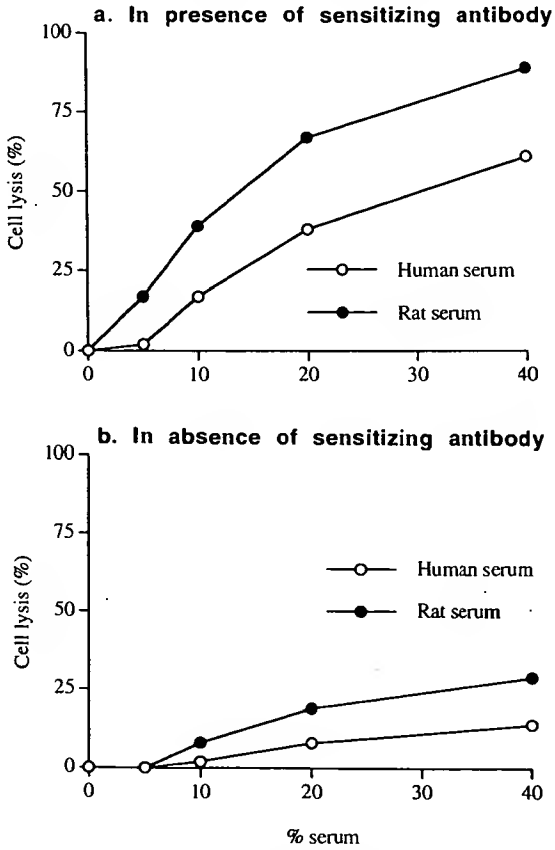

Fig. 1. Lysis of LAN-1 cells by human and rat complement. LAN-1 cells were incubated in the indicated concentration of rat or human serum in either the presence (*a*) or absence (*b*) of anti-GD2 complement-activating antibody (3F8 mAb). Complement-mediated cell lysis was determined after a 1-h incubation at 37°C. Representative data from at least three experiments are shown.

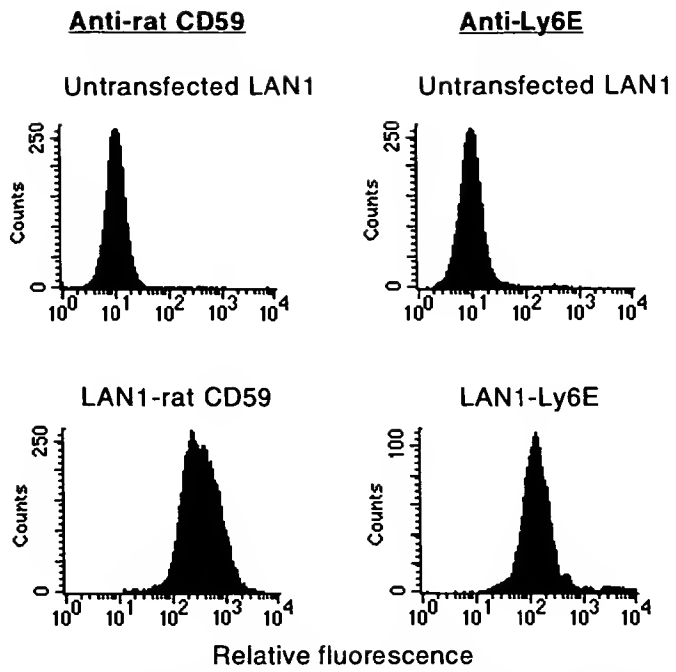

Fig. 2. Expression of rat CD59 and Ly6E on transfected LAN-1 cells. Stably transfected homogenous populations of LAN-1 cells expressing rat CD59 or murine Ly6E were isolated by several rounds of cell sorting. The figure shows flow cytometric analysis of sorted populations. Cells were stained by immunofluorescence using antirat CD59 mAb (6D1) or anti-Ly6E mAb (D7). Histograms of the relative fluorescence intensities are shown.

Expression of Rat CD59 on LAN-1 Enhances Tumorigenicity in Nude Rats. We first determined the tumorigenicity of LAN-1 cells in immune-deficient rats. The result of a dose-response experiment after s.c. injection of LAN-1 cells into the flank of nude rats is shown in Table 1. To investigate the effect of CD59 expression and increased complement resistance on *in vivo* tumor growth, control-transfected LAN-1 cells and LAN-1 cells stably expressing rat CD59 were injected separately into nude rats, and tumor growth was monitored. Groups of nude rats were inoculated with either 8×10^6 cells, a dose resulting in almost 100% tumor take for untransfected LAN-1 cells, or 4×10^6 cells, a dose determined to result in tumor growth in approximately 50% of animals (Table 1).

When LAN-1 cells expressing rat CD59 were injected into nude rats at a dose of 4×10^6, 100% of rats grew tumors, and the onset of tumor growth was earlier than that seen for control-transfected LAN-1 cells ($P < 0.01$, χ^2 analysis). Regression analysis showed that the rate of tumor growth was also significantly faster in rats inoculated with rat CD59-transfected cells ($P < 0.01$). In addition, analysis of the mean difference in tumor size on each day of tumor measurement between the two groups of rats showed that tumors growing in rats inoculated with rat CD59-transfected cells were significantly larger, with Ps < 0.01 and an average P value of 0.0021 (Student's t test; Fig. 4*a*).

Increasing the inoculation dose to 8×10^6 cells resulted in almost 100% tumor take with both rat CD59-transfected cells (19 of 19 rats) and control cells (19 of 21 rats), as expected from the dose-response data shown in Table 1. The onset of tumor growth, however, occurred significantly earlier in rats inoculated with rat CD59-transfected cells (Fig. 5); the mean day of tumor onset was day 13 for rats inoculated with control LAN-1 cells and day 7.4 for rats inoculated with rat CD59-transfected LAN-1 cells. One week after inoculation, 7 of 21 rats inoculated with control LAN-1 cells contained tumors, whereas 15 of 19 rats inoculated with rat CD59-transfected LAN-1 cells contained tumors. This is a highly significant difference ($P = 0.001$, χ^2 analysis). Similar to the data obtained with an inoculum of 4×10^6 cells, there was also a highly significant difference in the mean tumor size between rats inoculated with either control or rat CD59-transfectants at each day of tumor measurement, with Ps < 0.01 and an average $P < 0.001$ (Student's t test; Fig. 4*b*).

Although there was a highly significant difference in the rate of tumor growth between rat CD59- and control-transfected LAN-1 cells when rats were inoculated with 4×10^6 cells, there was a less pronounced difference in rats inoculated with a higher number of cells (compare Fig. 4, *a* and *b*). In this context, our data indicate the presence of low concentrations of natural endogenous antibodies in nude rats that bind to LAN-1 cells (see above), and when a high cell inoculum or after a threshold tumor size is reached, it is possible that endogenous antitumor antibodies may become depleted. At this point, complement may no longer be effectively activated at the tumor cell surface, and complement-sensitive (control-transfected cells) and -resistant cells (rat CD59-transfected cells) may grow at similar rates.

Complement Deposition and Expression of Complement Inhibitors on Tumor-derived LAN-1 Cells. Cells isolated from tumors after 28 days of growth were initially analyzed for deposition of

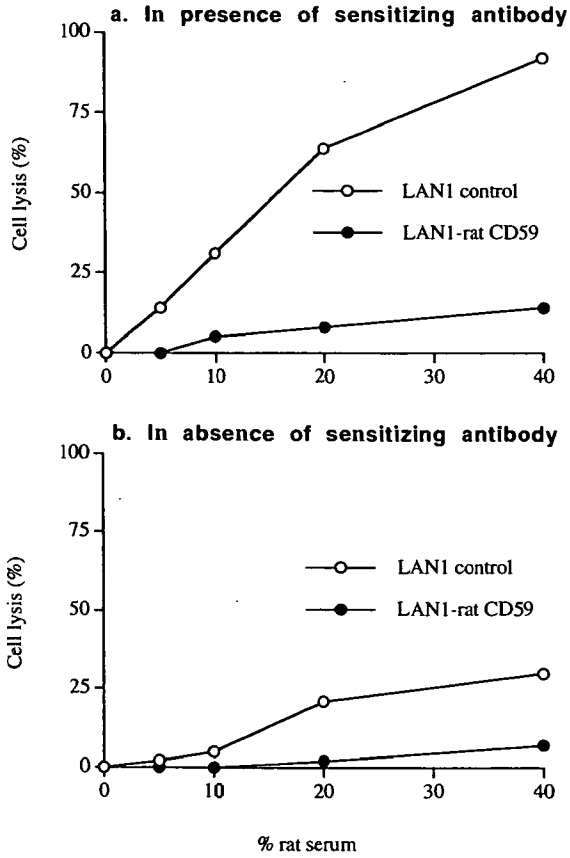

Fig. 3. Rat complement-mediated lysis of LAN-1 cells and LAN-1 cells expressing rat CD59. LAN-1 cells or LAN-1 cells stably expressing rat CD59 were incubated in the indicated concentration of rat serum in either the presence (*a*) or absence (*b*) of anti-GD2 complement-activating antibody (3F8 mAb). Complement-mediated cell lysis was determined after a 1-h incubation at 37°C.

Table 1 *Tumor incidence of LAN-1 cells in immune-deficient rats*

No. of cells injected[a]	No. of rats with tumor/ no. of rats inoculated
1×10^7	8/8
8×10^6	9/10
4×10^6	4/10
1×10^6	0/6

[a] Rats were inoculated s.c. in the flank and examined for tumor growth for up to 30 days.

complement and the continued expression of transfected rat CD59 by flow cytometry. As shown in Fig. 6, expression of rat CD59 was maintained on the tumor cells at a level similar to that seen in *in vitro* cultured cells used for inoculation. Interestingly, the level of Ly6E expression on control-transfected LAN-1 cells was not maintained during *in vivo* growth. This finding may be the result of selective pressure exerted by rat complement on rat CD59 expression.

As shown above (see Fig. 1), unsensitized LAN-1 cells are lysed by rat complement *in vitro*, and, as anticipated, complement proteins C3 and C9 were both deposited on LAN-1 tumors *in vivo*. Less deposited C9 was detected on rat CD59-transfected tumor-derived cells than on tumor-derived control LAN-1 cells (Fig. 6), consistent with the known function of CD59. More surprising was the finding that rat CD59-transfected tumor cells also had lower levels of C3 deposited on their surface as compared with control cells; the difference was small but consistent (Fig. 6 shows the results from a representative analysis). This was surprising because CD59 does not inhibit complement activation and is not expected to influence C3 deposition. An explanation for these data was provided, however, when we analyzed the endogenous expression of complement inhibitors on LAN-1 cells. We compared the relative levels of endogenously expressed DAF, MCP, and CD59 between *in vitro* cultured LAN-1 cells and LAN-1 cells isolated from tumors. Fig. 6 shows that DAF expression was up-regulated on the surface of tumor-derived control LAN-1 cells by about twofold compared with *in vitro* cultured cells. The relative level of DAF expressed on rat CD59-transfected cells derived from tumors was even further up-regulated compared with that in cells grown *in vitro* (about threefold). Thus, the increased level of DAF expression is likely to account for the decreased level of C3 deposited on the rat CD59-transfected tumor-derived cells. Of relevance to this finding, human DAF is known to inhibit rat complement, albeit less effectively than human complement (see "Discussion"). Multiple tumors from separate experiments were analyzed by flow cytometry, and the data shown in Fig. 6 are representative of at least six determinations for particular antigen groups. Transfection of LAN-1 with rat CD59 did not alter the level of endogenous DAF expression on cells cultured *in vitro*, and the level of endogenous CD59 and MCP expression on LAN-1 cells was unchanged after *in vivo* growth (Fig. 6). It is unlikely that the increased levels of DAF on LAN-1 cells after *in vivo* growth are due to selection because populations expressing higher-than-normal amounts of DAF could not be selected by cell sorting *in vitro*, and selection is not consistent with the finding that even higher levels of DAF are seen on rat CD59-expressing cells grown *in vivo*.

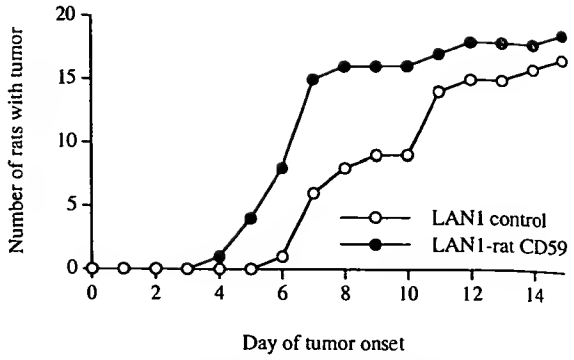

Fig. 5. Effect of rat CD59 expression on the onset of LAN-1 tumor growth. Control LAN-1 or rat CD59-transfected LAN-1 cells (8×10^6) were injected s.c. into the flank of nude rats, and the rats were examined daily for the appearance of a tumor (tumor recorded at a minimum diameter of 0.25 cm). $n = 21$ for the control group, and $n = 19$ for the rat CD59-transfected group.

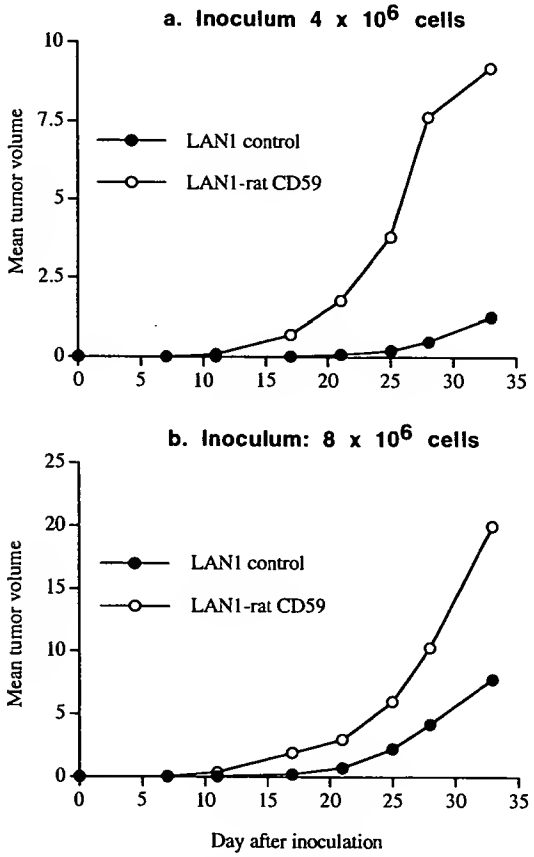

Fig. 4. Growth curves of control LAN-1 cells and rat CD59-transfected LAN-1 cells in nude rats. Either 4×10^6 cells (*a*) or 8×10^6 cells (*b*) were injected s.c. into the flank of nude rats. Growth was measured at intervals for 33 days. For experiment with a 4×10^6 inoculum (*a*), $n = 8$ rats/group; for the 8×10^6 inoculum (*b*), $n = 19$ rats/group.

DISCUSSION

It has been hypothesized that complement inhibitors on the surface of tumor cells present a barrier to immune-mediated clearance of tumor cells by contributing to the ineffectiveness of humoral immune responses observed in some cancers or by preventing effective mAb-mediated immunotherapy. Nearly all human tumor cells examined express membrane complement-inhibitory proteins, and most display a high level of resistance to lysis by human complement *in vitro*, even in the presence of antitumor complement-activating antibodies. On the other hand, human tumor cell lines are more susceptible to lysis by heterologous complement. We show here that the LAN-1 human neuroblastoma cell line is highly susceptible to lysis by rat complement, despite the endogenous expression of complement-inhibitory proteins. Of relevance to this finding, we have shown previously that human CD59 is not an effective inhibitor of rat complement (6). Here, we established a LAN-1 neuroblastoma cell line stably expressing rat CD59 for use in a rat model of human cancer relevant for studying the role of complement and complement inhibitors. Using this model, we demonstrate directly that a complement inhibitor expressed on the surface of a tumor cell can influence tumor growth. We also found that DAF was up-regulated at the LAN-1 tumor cell surface after growth *in vivo* and that DAF was even further up-regulated on tumor cells expressing functional (rat) CD59 when grown *in vivo*. Increased DAF expression was associated with decreased C3 deposition. These data demonstrate that the expression of complement inhibitors on a tumor cell has functional consequences with regard to complement deposition and tumor growth.

The expression of membrane-bound complement-inhibitory pro-

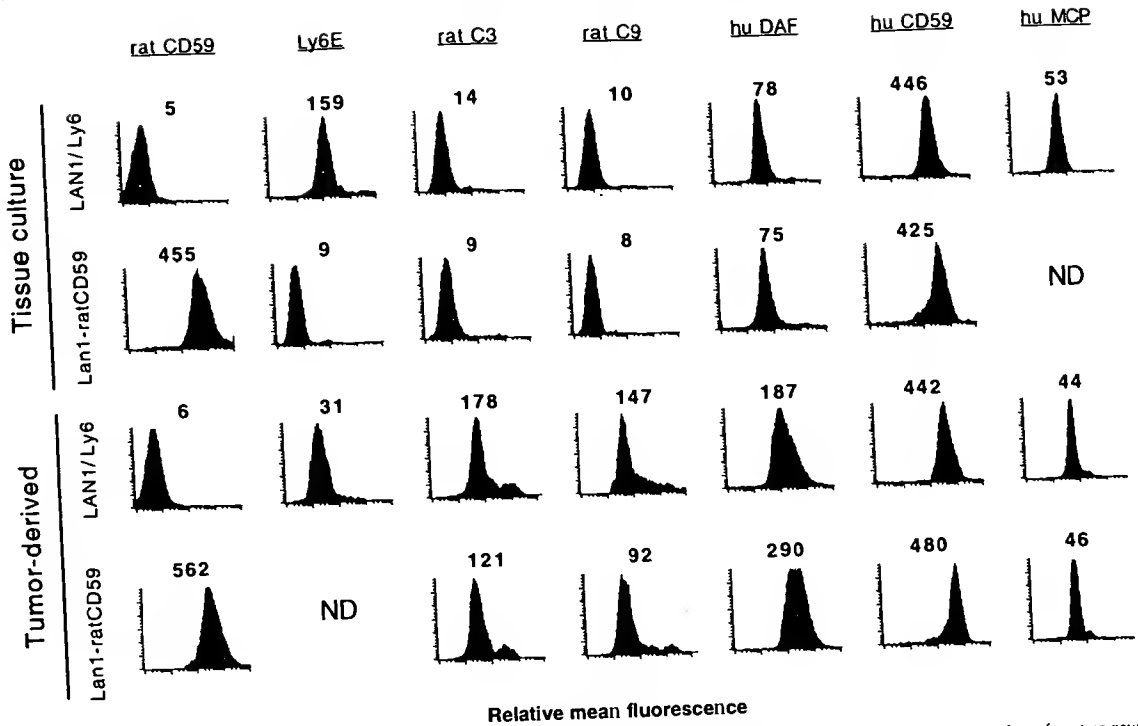

Fig. 6. Flow cytometric analysis of LAN-1 and rat CD59-transfected LAN-1 cells. Control or rat CD59-transfected LAN-1 cells grown in tissue culture (*top two rows*) or cells isolated from tumors (*bottom two rows*) were analyzed for expression of complement inhibitors and for the deposition of complement proteins as indicated. Cells were stained by immunofluorescence using appropriate antibodies (see "Materials and Methods"). The figure shows histograms of relative fluorescence, with *numerals* indicating the relative mean fluorescence intensities. Representative data are shown from at least six separate analyses for each antigen.

teins may benefit tumor cells for several reasons. Complement activation products (particularly C5a and the MAC) are powerful mediators of inflammation and may promote the recruitment of immune effector cells to the site of tumor growth. Cell-bound C3 activation products can promote and enhance antibody-dependent cell cytotoxicity and natural killer effector systems, and formation of the MAC can be directly cytolytic. Therefore, at least conceptually, it is reasonable to consider that up-regulation of complement inhibitors, as we observe here for DAF, may represent a mechanism by which some tumors can escape immune destruction. DAF is an inhibitor of complement activation and will inhibit the generation of C3/C5 activation products as well as the terminal MAC, whereas CD59 inhibits only MAC assembly. Because of the effect of CD59 on DAF expression, the current data do not provide information on the relative roles that these mechanisms may play in controlling tumor growth. However, the data do clearly establish that complement is involved in controlling tumor growth in this model and that CD59 promotes tumor growth whichever complement-associated mechanism(s) is operative.

So how does *in vivo* growth and, in particular, the expression of functional CD59 modulate DAF expression? Complement activation products and various cytokines have been reported to modulate complement inhibitor expression *in vitro*, although the effects appear to be variable, particularly for DAF (3, 4, 16–21). Also, a recent *in vitro* study reported that assembly of the MAC on endothelial cells directly up-regulated DAF expression and that expression was enhanced by cytokines (16). Similar mechanisms may be responsible for the up-regulation of DAF on tumor cells *in vivo*, as reported here. To explain the higher levels of DAF observed on rat CD59-expressing LAN-1 cells derived from tumors, it is conceivable that CD59-expressing cells may be able to survive higher levels of MAC that are initially deposited on the cell surface, thus enhancing the signal for DAF expression. CD59 limits the number of C9 molecules bound per MAC, and complexes containing bound C9, but with abrogated lytic function, may still be able to provide the signal for DAF up-regulation. It is also possible that the signal for induction of DAF expression is delivered via rat CD59 after its engagement by assembling complement complexes. This notion is consistent with the demonstration that CD59 is a signal transducing molecule (22–26). Increased endogenous DAF expression on LAN-1 cells correlated with decreased rat C3 deposition, and in this regard, human DAF is able to inhibit rat complement, although it is a less effective inhibitor of rat complement than human complement.[4]

We show that LAN-1 cells activate rat complement in the absence of exogenously added complement-activating antibody both *in vitro* and *in vivo*. This is probably due to the presence of natural endogenous xenogeneic antibodies because rat immunoglobulin is deposited on the LAN-1 cell surface after the incubation of cells in nude rat serum. It may be that tumor cell lines that do not "spontaneously" activate rodent complement will require the administration of exogenous complement-activating antitumor antibodies for an effect of complement inhibitors on tumor growth to be observed in rodent hosts. Indeed, human tumor cell lines transfected with rodent complement inhibitors and grown in rodents may represent good preclinical models relevant for evaluating tumor-specific mAbs. For our studies, we chose to use a rat model because the rat complement system appears to be more robust than the murine complement system and may represent a better model. It is difficult to isolate hemolytically active mouse complement, and there are reports documenting low complement levels in common laboratory mouse strains and nude mouse strains as compared with complement levels found in humans and rats (27, 28).

In summary, our results show that a membrane complement inhibitor expressed on the surface of a tumor cell plays a role in determining tumorigenesis and that reversing the effects of tumor-specific complement regulators is likely to enhance immune-mediated clear-

[4] C. L. Harris, O. B. Spiller, and B. P. Morgan, personal communication.

ance of some tumors. The widespread expression of membrane-bound complement inhibitors presents technical difficulties for the selective blocking of complement inhibitors on tumor cells. However, it may be possible to adapt current and developing technologies to permit targeted delivery of antibodies, peptides, or perhaps antisense DNA to block the effects of endogenous complement inhibitors expressed on tumor cells.

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